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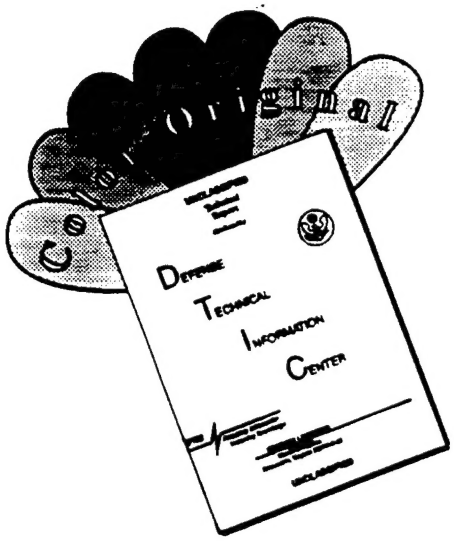

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Role of Matrix Metalloproteinases and Their Tissue Inhibitors in Human Breast Adenocarcinoma

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ABSTRACT

The high fatality of breast cancer is mostly caused by metastases. Excessive secretion of a group of matrix metalloproteinases (MMPs) or decreased production of their tissue inhibitors (TIMPs) has been implicated in tumor invasion and metastasis. The hypothesis that an imbalance of MMPs over TIMPs may contribute to the invasive and metastatic processes of human breast tumors was tested by localizing and contrasting the expression of specific MMPs; gelatinases, collagenases and stromelysin and their inhibitors (TIMPs, -1, -2, & -3) by immunohistochemical analysis of tissue sections of human breast carcinoma. MMPs and TIMPs were quantitated from the same tissue used for immunohistochemistry by gelatin substrate zymography (MMPs, gelatinases), reverse zymography (TIMPs) and substrate protein assays (collagenases, MMP-1). Gelatinases can be quantitated easily from small amount of tissue by zymography using an imager and GelBase Pro software. Zymography of tissue extracts showed that MMP-9 was exclusively found in breast carcinomas in varying amounts (11 specimens) and enzyme levels were 15-30 times higher than that found in normal and benign tissues. This finding was confirmed by immunostaining of tissue with specific MMP antibodies. Several tumor cell lines (MCF-7) showed the secretion of mainly MMP-9.and this was enhanced by stimulation of cells with phorbol ester. Immunofluorescence staining and confocal analyses of stimulated cells showed the presence MMP-9 and MMP-2 (not secreted in the media) around the cell plasma membrane. These results are of importance specially with the finding of membrane bound MMP. Reverse zymography of tissue extracts showed the presence of TIMPs,but at this point quantitation was not acheived but feasible in future.

FOREWORD

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Ernest Grimsley Smith 9/27/95

PI - Signature Date

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ROLE OF MATRIX METALLOPROTEINASES AND THEIR TISSUE INHIBITORS in HUMAN BREAST ADENOCARCINOMA

(5) INTRODUCTION

BACKGROUND

5.A.1. Breast Cancer

The overall objective of this research is to explore the role of matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) in the invasive and metastatic processes of human breast tumors [1-4]. The hypothesis to be tested is that an imbalance arises through normal or elevated production of MMPs accompanied by a diminished production of inhibitory TIMPs. Metastasis of an initially localised tumor to vital organs is the dominant cause of cancer related deaths [5]. The mechanisms controlling the metastatic progression of a localized tumor are very complex, involving many biochemical and cellular events [6]; but recent evidence indicates that secreted matrix metalloproteases (MMPs) play a major role in the penetration of the basement membrane surrounding the tumor [7].

Metalloproteinases are needed for invasiveness of tumors. The most important traits of tumor cells are their ability to specifically attach to the extracellular matrix (ECM), degrade this extracellular barrier so as to escape out of the primary location into the circulation and establish themselves at the site of metastasis. The breakdown of the ECM can be traced to the action of one or more members of the family of zinc proteases named **matrix metalloproteinases (MMPs)** secreted as proenzymes [8] and are activated outside the cell by serine proteases such as plasmin [9-11] by the removal of the 9 kDa prosegment from the active site. Three enzymes are most likely responsible for the degradation of ECM [5, 11]: 72 kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), 92 kDa gelatinase (gelatinase B, type V collagenase, MMP-9), and the 57 kDa stromelysin (MMP-3) [12-15]. 72 kDa gelatinase was implicated in human breast cancer where 36/40 cases of invasive tumors were positive by immunohistochemistry [16]. Stromelysin and also interstitial collagenase (MMP-1) have been implicated in human breast cancer by Polette [17] and Clavel [18]. Recently, stromelysin-3 has been found in breast cancers [19], but the proteolytic activity of this enzyme appears to be very weak [20].

5.A.2. Metalloproteinase action is limited by specific inhibitors

In addition to MMPs, cells also produce TIMPs. These are small proteins that neutralize the destructive activities of MMPs and play an important role in controlling ECM degradation. There are at least three species that may be implicated in tumors: TIMP-1, TIMP-2 and TIMP-3 of relative mass 28 kDa, 20 kDa, and 23 kDa respectively [9]. They bind to activated MMPs in a 1:1 molar ratio and inhibit their activity. TIMP-2

and TIMP-3 share an amino acid homology with TIMP-1, although they are encoded by different genes [21]. Moreover, many cells produce MMP-2 in a 1:1 complex with TIMP-2; similarly, MMP-9 usually comes with an associated molecule of TIMP-1. The TIMP molecule in these cases is bound to the C-domain of the enzyme, but interferes with the activation of the latent gelatinases (MMP-2and MMP-9). The role of TIMP-3 is just begining to unravel. TIMP-3 binds to ECM and is difficult to solubilize and its role in cancer has not been identified. Although some reports [22] show that TIMP-3 is secreted during normal differentiation processes in normal mouse embryos and may play a role in the development of cancer *in vivo* and may be secreted in the early cancer development [23].

5.A.3. Role of MMPs and TIMPs in breast cancer

Since normal cells as well as non-invasive cells produce MMPs and also TIMPs, it seems likely that the extent of activation of MMPs and the levels of inhibitory TIMPs may be two key factors in the progression of normal to invasive cell type. Elevated levels of MMP-9 have been detected in the plasma of patients with breast cancer [24] although no such correlation was found for MMP-2 [25].

Not all of the MMPs and TIMPs need be related to the tumor cells. It is quite likely that tumor cells can stimulate neighboring stromal cells through cytokines to produce enzymes that degrade the matrix. So in certain breast tumors and other cancers of skin and colon, 92 kDa gelatinase was found at the tumor/stroma border but not in tumor cells [4].

5.A.4. Summary of Background

In summary, there is compelling evidence of a role for metallo-proteases (and other proteases) and the TIMP inhibitors in tumor cell invasiveness. There are lines of evidence to support the concept that an increase in gelatinases and a decrease in TIMPs may be important in this process together with a greater conversion of latent to active forms of the MMPs. The resultant increased MMP activity would permit penetration of basement membrane surouding the tumor by cells which produce these enzymes or stimulate neighboring stromal cells to produce such enzymes. The same processes would also be important in metastasis of the tumor cells. What is generally missing from these literature reports is a unified study of the changes of multiple enzymes and inhibitors in the same tissue, bolstered by immunohistochemical data on enzyme localization, biochemical assay of latent and active enzyme activities and molecular biological determination of mRNA levels. It is the development of such a unified picture. We have begun this unified study and our first year research report attests to our ability to perform such studies.

5.b. PURPOSE OF THE PRESENT WORK:

The underlying hypothesis to be tested is that the invasiveness of human breast cancer is dependent on the action of specific metalloproteinases that can degrade the constraining basement membranes of the extracellular matrix. This action, in turn, depends on two factors - how much active form of enzyme is present and how much inhibitory TIMP is present. Our hypothesis is that an imbalance occurs such that invasive cells produce (or cause to be produced) more active enzyme and less inhibitor than normal cells or cells of benign tumors.

In order to test the hypothesis, the following types of studies are planned. i) Identification and localization of various MMPs and TIMPs by immunohistochemistry, with qualitative grading and measurement of enzyme activity by the use of substrates for gelatinase and stromelysin activities. ii) Determination of the amounts and ratios of active and latent enzyme by substrate zymography and immuno-precipitation combined with zymography. Methods suitable for extracting and assaying enzymes directly from tumor tissue are available. iii) Determination of mRNA levels for the MMPs and TIMPs in the same tissue samples, based on Northern blotting. iv) Culture of epithelial cells growing out from explants of human mammary carcinomas and determination of their production of active and latent MMPs and TIMPs.

The research study takes advantage of unusual resource available to us - a repository or archive of tumor tissue collected for 4 years complete with sections and snap-frozen blocks of fresh tissue. Because of the existence of the Florida tumor registry, it is possible to return to these tissues, to examine them by immunohistochemistry and then to perform biochemical measurements on the frozen blocks of tissue. The MMPs and TIMPs are extremely stable, so there is no problem in measuring after prolonged storage. One can then use the results in prognostic fashion, since it is known if the patients subsequently developed metastasis, or died of the cancer.

5.C. METHODS OF APPROACH

Identify, localize and contrast the the expression of specific MMPs and TIMPs by immunohistochemical analysis of tissue sections of human breast carcinoma

Initially, the plan is to investigate tissues classified by pathological evaluation: i) Invasive carcinoma with no subsequent metastasis ii) invasive carcinoma with subsequent metastasis and iii) **in situ** carcinoma with no metastasis. These tissues will all be drawn from a group collected and stored as both paraffin blocks and frozen cubes of tissue (1 cm^3). The subsequent history of the patients can be determined from the registry. Sections will be stained with one of the 6 monospecific polyclonal rabbit antibodies to MMPs and TIMPs and examined for didstribution of reaction, relation to cell interior/exterior and cell type, intensity, etc. As the data collection continues, the groups expanded and make finer subdivisions to include

age, estrogen status, lobular versus ductal location, nuclear grade, etc.. This can be done in subsequent years. The tissue archives will, of course, continue to grow with each subsequent year. We do not anticipate many difficulties with this aim. The antibodies used will be checked periodically for reactivity by Western Blots.

Identify, characterize and quantitate the MMPs and TIMPs and their mRNA from the same tissue used for immunohistichemistry: Enzymes, inhibitors and their mRNAs are extracted from small frozen blocks of tissue, corresponding to sections examined for enzymes and immunohistochemistry. We have developed methods for protein extraction that are quantitative. We also have developed assay methods over many years that are quite sensitive, detecting subnanogram amounts of MMPs and TIMPs. These methods are detailed in the next section. In addition to enzyme assays, extensive use will also be made of zymography for detecting MMPs and of reverse zymography for detecting TIMPs. These methods permit estimation of the active and latent forms of MMPs (since both forms display activity upon refolding from SDS) and distinction of TIMP-1 and -2. The gelatinases present a special problem in zymography (overlapping bands) which can be solved by a new method of immunoprecipitation followed by zymography. Finally, the mRNA levels are to be determined by Northern analysis using specific cDNA probes. If necessary RT-PCR methods can be used These various approaches should enable us to test our hypothesis about enzyme/inhibitor imbalance in several ways.

Isolation and culture of primary human mammary epithelial cells from breast cancer tissue explants.

Using methods by which we successfully isolated the primary epithelial cells from prostate tissues [26], we will do the same for human carcinomas. We cannot use the archival tissue in this study, but will obtain fresh benign and invasive tumor tissues. The resultant cells growing out in the epithelial cell selection medium will be compared for production of TIMPs and latent and active MMPs. The conditioned media will be analyzed by zymography, reverse zymography, quantitative enzyme assays, and ELISAs. Enzyme and inhibitor activities will be corelated to protein content of the media or to the cell numbers.

(6). **BODY**

6.A EXPERIMENTAL METHODS.

6 A.1.Tissue Samples: The tissues (~ 1 cm^3, snap frozen, stored at -70° C) diagnosed as displaying breast carcinoma, normal or begnin (fibrosarcoma) are obtained from the Tissue Procurement laboratory, Pathology Department, University of Miami Medical School, Miami Florida. Corresponding to each frozen sample is a paraffin-fixed block of adjacent tissue which are used for immunohistochemistry. In each subsequent year, a new series of tissues are available. The tissues chosen for present study were chosen randomly that is tissues from years 1991-94 with or without follow-ups (history) and were not classified into subtypes.

In the second year onwards the tissues will be chosen : 1) Specimens showing tumors histo-pathologically evaluated as invasive with the donor patients showing no subsequent metastatic disease. 2) Specimens showing tumors histo-pathologically evaluated as invasive with the follow up of the patients showing development of metastatic disease. 3) Specimens showing tumors histo-pathologically evaluated as noninvasive **in situ** tumors showing no subsequent metastatic disease.

The Florida tumor registry can provide the history of patients in intervening years regarding the development of metastasis, survival rate of the patients up to five years and beyond. The snap frozen tissues kept for five years will be suitable for Northerns (mRNA estimation) as long as care is taken during the preparation of total tissue RNA [27,17].

Informed consent forms are not needed for this study. In accordance with 21 CFR 56.111(a)(3), expedited review (#95/480) has been approved by the University of Miami's Human Subjects Institutional Review Board for use of residual tissues.

6.A.2. Explant cultures: Tissues either fresh or frozen in 5% dimethyl sulfoxide are obtained according to State and Federal regulations from the Tissue Procurrement Laboratories, Sylvester Cancer Center, University of Miami. Neoplastic tissues are obtained as chips or pieces and classified for tumor grades by the University of Miami Pathology Reference Service.

Tissues are collected under sterile conditions, minced into pieces, rinsed (PBS), seeded [26] in T25 flasks and cultured in MEGM - a serum free epithelial growth medium (Clonetics Corp., San Diego, CA). The conditioned medium(CM) is collected, filtered and stored in aliquots at -70°C until used for enzyme and TIMP analyses.

The epithelial nature of the cell monolayer is confirmed by immunocytochemical staining, using an anti-cytokeratin monoclonal antibody specific to cytokeratin 8 and 18 (CAM 5.2, Becton-Dickinson Immunocytometry Systems, San Jose, CA). Greater than 90% of the cells should stain positive in randomly selected microscopic fields under observation [26].

6.A.3. Cell culture model and Cell lines: A number of breast carcinoma, prostate carcinoma and other carcinoma cell lines are screened for their ability to synthesize and secrete MMPs and TIMPs in culture.Collection of **MCF-7 , DA-3, MDA-MB-231, HT 101 (supplied by Goodwin Institute, Florida), PC-3 and PPC-1 provided by Dr. Gina Bai, Univ. of Miami** or other mammary tumor cell supernatants. Tumorigenic epithelial cell line **MCF-7** (human breast carcinoma) and non tumoregenic cell line **CV-1** (isolated from normal monkey kidney) are provided by **Dr. T.J. Lampidis**, Dept. of Cell Biology & Anatomy, Univ. of Miami Med. Sch. **DA-3** cell suspensions will be provided by **Dr. Diana Lopez**, Dept of Micro. & Immunology, Univ. of Miami.

The cell lines are maintained as cell suspensions in DMEM-F12 supplemented media (5 - 10% FCS), passaged as necessary and after confluency, the cells are maintained in serum free media for 1, 2, 4, 24 or 48 h when media is separated and frozen at -70^{o}C for various biochemical analysis. The secretion pattern of MMPs and TIMPs of these established cell lines are compared with the secretion pattern from the tumor tissue extracts. The cell lines are also treated with phorbol 12-myristate 13-acetate (PMA), cytokines, growth factors and inhibitors.

6.A.4.Generation of antibodies (IgGs) and western blotting: Antibodies were raised in rabbits against the whole molecule of enzyme stromelysin (MMP-3 from human cartilage), TIMP-1 from human cartilage chondrocytes, TIMP-3 from ECM of MCF-7 cultures and TIMP-2 from skin fibroblasts. Pro-segment peptides for 92 kDa, 72 kDa, and stromelysin (MMP-1), metal binding region peptides of 92 kDa and 72 kDa and N-terminal peptides of active enzyme, MMP-1 (interstitial collagenase) containing 15-17 amino acids were prepared by the Peptide Laboratories of Department of Biochemistry and Molecular Biology using Advanced Chemtech automatic synthesizer model ACT350. These purified peptide preparations were conjugated to bovine serum albumin, ovalbumin or hemocyanin, dialyzed, and injected into rabbits to raise the antibodies [28].

The polyclonal antibodies were found to be monospecific. They were further characterized for their reactivity to other MMPs with the use of Western blot analysis [29] and ELISA method [28].

6.A.5. Immunohistochemistry: These analyses are carried out by Dr. Mehrdad Nadji, Co-Investigator, Department of Pathology, University of Miami Medical School. Paraffin sections (3 microns) are cleared of paraffin, blocked for endogenous peroxidase, washed in water, PBS, blocked with normal horse serum and then are treated with drops of specific primary antibodies in a humidity chamber (1-3 h). The tissues are washed, and treated with a biotinylated secondary antibodies (1-3h), followed by avidin-biotin-peroxidase complex [30,4]. They are then washed and treated with the chromogen (DAB, 3-3'-Diaminobenzidene). The slides are counterstained with hematoxylin, washed, dehydrated and evaluated for the localization of various MMPs and TIMPs in specific cells in the tissue.

6.A.6.Extraction of Metalloproteinases. Tissues are extracted with Triton-X 100 (to solubilize membranes) followed by 1-2M GuHCl. In the human heart [31] and uterus studies [32] indicate that 2 M GuHCl or 0.25% Triton-X 100 extracted 90-95% of all MMPs and TIMPs.

Human breast tissues are weighed (100-200 mg), minced (finely) and homogenized (in the hood) in 7.5 volumes extraction buffer (0.25% Triton-X 100 or 2 M GuHCL in 50 mM Tris/HCl buffer, pH 7.5 using Polytron homogenizer, centrifuged and the pellet reextracted with 2.5 volumes of appropriate extraction buffer, centrifuged and supernatants combined. All steps are carried out at about 4^{o}C. Extracts are dialyzed and stored in aliquots at -70^{o}C. The extracts were fractionated by column chromatography using ACA54 molecular seive resin [29] or directly used for zymography and other techniques.

Most tissues contain inhibitory activity which appears to be TIMP (tissue inhibitor of metalloproteinases). These can be destroyed, without affecting the metalloproteinases, by reduction (2 mM DTT) and alkylation [33]. This step also destroys any alpha-2-macroglobulin. 5 . DNA content of tissue homogenates are measured by the method of LaBarca and Paigen [34]. Protein estimation of homogenates or concentrated media are by the use of BioRad protein estimation kit.

6.A.7. Enzyme and inhibitor assays: Tissue extracts (with or without reduction and alkylation) and column fractions (molecular seive or affinity chromatograophy) are assayed and quantitated for various MMPs using (1) ^{3}H-acetylated Type I gelatin to estimate the MMP-2 and MMP-9 gelatinases [35] (2) ^{3}H-acetylated Type I rat skin collagen for MMP-1 (interstitial collagenase [36] and ^{3}H-carboxymethylated transferrin [37] ^{3}H-acetylated proteoglycan monomer bead assay [30] for stromelysin (MMP-3) and MMP-7. Blanks are set up with 1,10-phenanthroline and p-aminophenylmercuric acetate (APMA) are used to activate latent enzymes.

Measurement of TIMPs in tissue extracts, media or column fractions are acheived by the inhibition of uterine MMP-7 and remaining activity assayed using Azocoll as substrate [33]. After centrifugation of undigested Azocoll, the absorbance of dye-released supernatant is measured. One mole of MMP-7 is assumed to bind with one mole of TIMPs. Quantitation of the latent and active forms of various MMPs and TIMPs

are also done by immunoassay (EIA) [28] using monospecific monoclonal and polyclonal antibodies made against the whole enzyme molecules or against specific peptides for each MMPs. I have generated several polyclonal antibodies against most MMPs and the TIMPs.

6.A.8.Zymography and Immunoprecipitation. Gelatin zymography follows a modified procedure of Herron et al. [38] for detecting picograms of MMP-2 & -9 and nanograms of other MMPs and proteases. SDS-PAGE is performed in 7.5% or 10% polyacrylamide (39) containing 0.33 mg/ml gelatin. The gels are then rinsed twice in 0.25% Triton X-100, and incubated (18 h, 37^{O} C in Tris-NaCl-$ZnCl_2$ -$ZnCl_2$,3 mM phenylmethylsulfonyl fluoride (PMSF) assay buffer. Gels are stained with Coomassie blue R 250. Both latent and active forms of gelatinases or other MMPs produce clear areas in the gel. The relative amounts of enzymes are also quantitated by densitometry of gels (and also dried gels in membranes).The imager used is supplied by Department of Biochemistry, University of Miami Medical School to scan and quantitate 250-500 gels generated per year. The monies allocated ($7,500) in the first year were spent to purchase a Pentium computer and printer to use the software compatible with the imager. The software was purchased from Ultra Violet Products (UVP) as GelBase/GelBlot Pro Software. The image from the imager is recorded on discs and each lane quantitated for enzyme spots.

Tissue are extracted with buffered 0.25% Triton X 100 or concentrated filtered media (5 ml) from cell lines are chromatographed on using column of Ultrogel AcA-54 [29]. Fractions (3 ml) are collected and analyzed for gelatinases or other MMP(s) content by zymography or substrate assay methods. Fractions containing MMP(s) are immunoprecipitated with rabbit anti-MMP(s) IgG(s) using protein A-agarose suspensions. Blanks are prepared with specific IgG alone, preimmune serum with enzyme fraction, and enzyme with protein A gels but no IgG. After the reacted agarose gels are washed, the immune complexes dissolved in sample buffer and analyzed by zymography for specific enzyme activity or for proteins by SDS-PAGE (for TIMPs) [40]. By this method, 90-95% of the antigen present is immunoprecipitated.

6.A.9. Reverse zymography: A modified method of Heron et al [38] is used to detect the TIMPs. Recently, a kit is availble to perform reverse zymography of tissue extracts and conditioned media from University Technologies Internationa Inc.Calgary Canada. The kit provides standards of TIMP-1, -2 and -3 and the media containing the enzymes. Conditioned media (or extract) is fractionated by SDS-PAGE [39] electrophoresis using 12,5% acrylamide , 0.75 mg/ml gelatin solution and the supplied media (0.1 ml) containing enzymes. After washing with 2.5% Triton X-100, the gel is incubated in Tris buffer (37^{O} C, and minimum gentle shaking) and stained with Coomassie blue solution to reveal cleared and uncleared area of the gelatin in the gel. Uncleared blue staining areas are revealed only if TIMPs are present. This is a very sensitive method revealing as little as 2 ng of TIMPs.

6.A.10. Enzyme Linked Immunoassays, ELISAs: This method will be used to quantitate MMPs and TIMPs in tissue extracts, serum free media of cell lines and column fractions of the tissue extracts or media. The method is based on an inhibition immunoassay (under nonequilibrium conditions) [28], uses purified polyclonal antiserum and detects 5-500 pmoles of MMPs and TIMPs . The antibodies generated for each MMPs and TIMPs are monospecific and do not crossreact. The sample values are read from standard curves produced by several commercially available computer programs. Total MMPs or TIMPs are calculated as ng/mg tissue or ng/ml media. The ELISA method is useful as a quick guide to the amount of MMPs and TIMPs in a sample, so that appropriate amounts of samples can be applied for quantitation of MMPs by zymography. This method will be useful for quantitating total TIMPs because TIMPs-enzyme complex is not quantitated by the enzyme-based inhibitor assay. The ELISA method, however, is not suitable for distinguishing active and latent enzyme forms.

6.A 11.. RNA extraction from tissue cell samples and Northern Blot Analysis:

Tissues aliquots or confluent cells that were fresh rapidly frozen in liquid nitrogen and stored at -70^{o}C are used. Total cellular RNA is prepared by the method of Chomczynski and Sacchi [41] using RNA 201 Stat-60 kit (Tel-Test Inc. Freendstown, Tx) Standard Northern blot technique is used [41]. Briefly, total RNA (3-10 µg) is electrophoresed through agarose-formaldehyde gels, transfered onto nylon membranes Nytran, Schleicher & Schuell) by capillary electrophoresis, followed by prehybridization of the membrane, and hybridization with the appropriate radiolabeled probe. Labelled probes are obtained using Prime-a-Gene random hexamer kit (Promega, Madison, WI and NEN [alpha-^{32}P]dCTP. Quantification of bands are performed using a Molecular Dynamics PhoshmoImager. Normalization of the amount of RNA loaded in each lane, cDNA probe for constitutively expressed actin or Glyceraldehyde phophate dehydrogenase are hybridized on the same blot.

cDNA clones are obtained from American Type Culture Collection, Rockville, Maryland. ATCC has listed human fibroblast collagenase in vector pSP64 by Rahmsdorf, collagenase IVA in vector pBR322 by Tryggvason, stromelysin 1 (pUN121, by Matrisian), stromelysin (MMP-3, transin, in pUN121 by Matriasin); stromelysin 2 (transin-2) in pUn121 by Matriasin), TIMP-1, tissue inhibitor of metalloproteinase 1 in pTZ by Willard and TIMP-2 (two clones) in pBluescript SK^{-} by Venter. cDNA clone for PUMP-1 is available from rat uterus (UMP, MMP-7) in our laboratory.The message of the various MMPs and TIMPs may be difficult to analyze due to low amounts. It may be necessary to use reverse transcriptase-polymerase chain reaction (RT-PCR) in some cases; if so, specific primers are used to cover distinctive regions of the message The RT-PCR method [42] although difficult may be ultimate method of choice.

6.B.RESULTS:

The first year (August 1994-August 1995) of the four year granting period has been successful in generating some meaningful data regarding the role metalloproeinases and their tissue inhibitors in breast adenocarcinoma. The results are presented in Figs 1-15 and Table 1-2. We have established that the MMPs can be identified and quantitated by substrate zymography using the Imager and the software GelBase/GelBlot Pro in small breast tissue samples. Staining of the tissue sections of corresponding breast tissues with anti-MMPs IgG are corelated with the biochemical findings (zymographic evaluations). This is in partial fulfillment of one of the Specific aims cited in the grant. These findings need to be confirmed with larger number of samples for meaningful stastical analysis.

Another approach to the problem was to show the spatial location of various MMPs and tissue TIMPs using confocal microscope. This study has led to the collaboration with Dr. L. Bourguognon, Department of Cell Biology and Anatomy , Univ. of Miami Medical School.and also a recipient of DOD breast cancer grant. The preliminary studies from this collaboration shows that there may be a link between isoforms of CD44 (transmembrane gycoprotein) and the secretion pattern of MMPs. Implication of CD44 in breast and prostate cancer has been established by severla workers [42,43] including the laboratory of Dr. Bourgougnon in Miami. The letter of collaboration from Dr. L Bourgougnon has been included in Appendix, p .

This CD44-MMPs study has been tested first in the cell lines. Using the breast cancer cell line **MCF-7**, we show a corelation of MMP-2 and CD44 in the cell membrane. Staining with anti-MMP-9 reveals enzyme inside the cell in granules and around the nucleus and some around the cell membrane. In contrast staining with anti-MMP-2 enzyme is seen around the membrane. Rat anti-CD44 also shows staining around the edge of the cell membrane. This findings are observed in both the resting and PMA stimulated state in the cells .

However, when stimulated (PMA, phorbol 12-myristate 13-acetate) cells are stained with anti-MMP-2, the staining is observed in a broad visible around the membrane. Rat anti-CD44 staining also shows the stimulation of CD44 by PMA. Why is MMP-2 not secreted into the media similar to MMP-9? Is it associated with CD44? and cannot be secreted. This mechanistic observation deserves further consideration and will be the future approach in next granting period. We are excited about this finding and may shed further light on the membrane bound MMP-2.

Another exciting observation with CD44 studies was that addition of anti-CD44v-III (CD44 isoform) in the media with and without stimulation with PMA showed that antiserum had caused and initiated the activation of the secreted 92 kDa MMP. This finding was not observed with anti-CD44 (standard form) or with anti-CD44v-VI another isoform) These findings are exciting and requires further research to evaluate the role of MMPs in the transmembrane association. CD44v-III [42] has been

implicated in breast cancer. This study will therefore be a part of the ongoing research in the coming year.

6.B.1. Quantitation and characterization of MMPs in breast tissue:

Breast tissues (characterized) were homogenised using Polytron in 10 volumes of extraction buffer containing Triton-X 100, followed by 1 M Guanidine hydrochloride, dialyzed in standard Tris buffer, aliquoted and frozen at -70^{O} C. The extracts were routinely analyzed for the presence of MMPs using substrates, gelatin and carboxy methylated transferrin. and 10% acrylamide for SDS-PAGE electrophoresis. . In our hands, casein gels were not as successful. Gelatin gels gave a good profile of the MMPs present in the tissue extracts or media. The inclusion of 3 mM PMSF in substrate gel incubation buffer should mask serine proteases and reveal bands cleared mainly by MMPs on a coommassie blue stained gelatin gel.. A typical profile of extracts analyzed by substrate is shown in **Fig 1**. The prestained protein standards help to identify the various MMPs present in the extracts.

Fig 1 shows that normal breast tissue extracts reveal clear bands at molecular weight corresponding to MMP-2 (72 kDa, gelatinase A) The latent enzyme band is at Mr 68 kDa and active enzyme band at 62 kDa. There are also minor bands at 92 kDa Benign tissue shows exclusively the bands for latent and active for 72 kDa. In sharp contrast, the breast cancer tissue shows the presnce of MMP other than 72 kDa. There are bands that correspond to latent and active bands of 92 kDa (gelatinase B, MMP-9). Bands are also observed at Mrs higher than 92 kDa. and at Mrs lower than MMP-2. MMP-1 and MMP-3 digest the gelatin substrate 1000 times lower than the two gelatinases. Also lower molecular weight active enzyme bands of both gelatinases can be found in the regions that MMP-3 and -1 are found. Higher than 92 kDa band can be attributed to either aggregates of active 72 kDa (~125 kDa) or lipocalin bound dimer 72 kDa. Band seen near the top of gel may be aggregate band of active 92 kDa or lipocalin bound dimer of 92 kDa.

There is a clear difference in the secretion pattern in of MMPs in normal, begnin and cancer breast tissue. The results therefore indicated that 92 kDa MMP is a predominant enzyme in cancer tissue. Several reports in literature have implicated the role of MMP-9 (92 kDa, gelatinase A) in breast and several other cancer tissues [44, 45].

Figs. 2-4 show the zymography (gel) profile scanned by the UVP imager and analyzed on the computer by the software "GelBase/GelBase Pro" (UVP, Cambridge, England). The program tracks cleared bands in each track giving a profile of peaks. The peaks are calculated in computer numbers. Fig. 2a & b show the tracking and results of the analyzed latent and active 92 kDa MMP. Known amounts either in protein or the unit of activity of various MMPs are analyzed with breast tissue extracts on the same gel. This allows the quantitation of MMPs in smaal amounts of biopsied breast tissue.

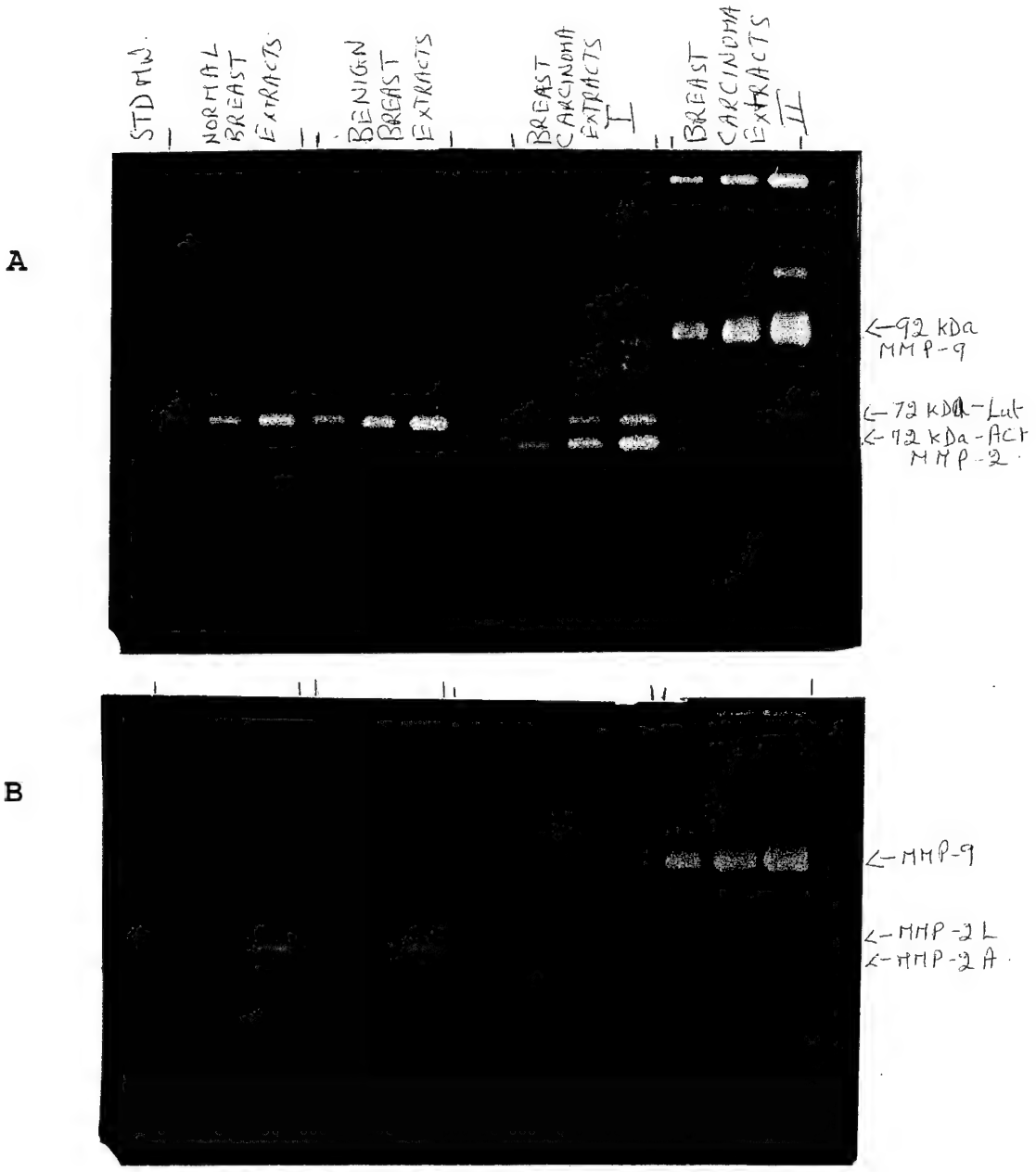


FIG. 1. Zymographic (gelatin) evaluation of breast tissue extracts. **A**: buffered 1M Guanidine hydrochloride; **B:** buffere 0.25% Triton X-100 extracts. 30-40% of total enzyme is found in Triton extracts.
MMP-9 (92 kDa gelatinase is not present in normal or benign breast tissue extracts. Breast carcinoma (infiltrating ductal) I & II tissue extracts show the presence of MMP-9. This finding is confirmed in the immunohistostaining of the same tissue with anti-MMP-9 IgG. MMP-2 is found in all tissue extracts.

03111519.DSK : Track 2 : Tue Sep 26 07: RESULTS

FIG. 2a

Peak	Rf/Mw/Bp	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos
1	N/A	2.96	N/A	97.64	3.00	8.88	3	33	69	164
2	N/A	17.58	N/A	6189.82	40.49	562.71	32	352	69	192
3	N/A	73.83	N/A	31675.08	164.59	2879.55	39	429	69	211

← PROMMP-9 PEAK

← PRO-MMP-9

BLANK

BREAST CARCINOMA

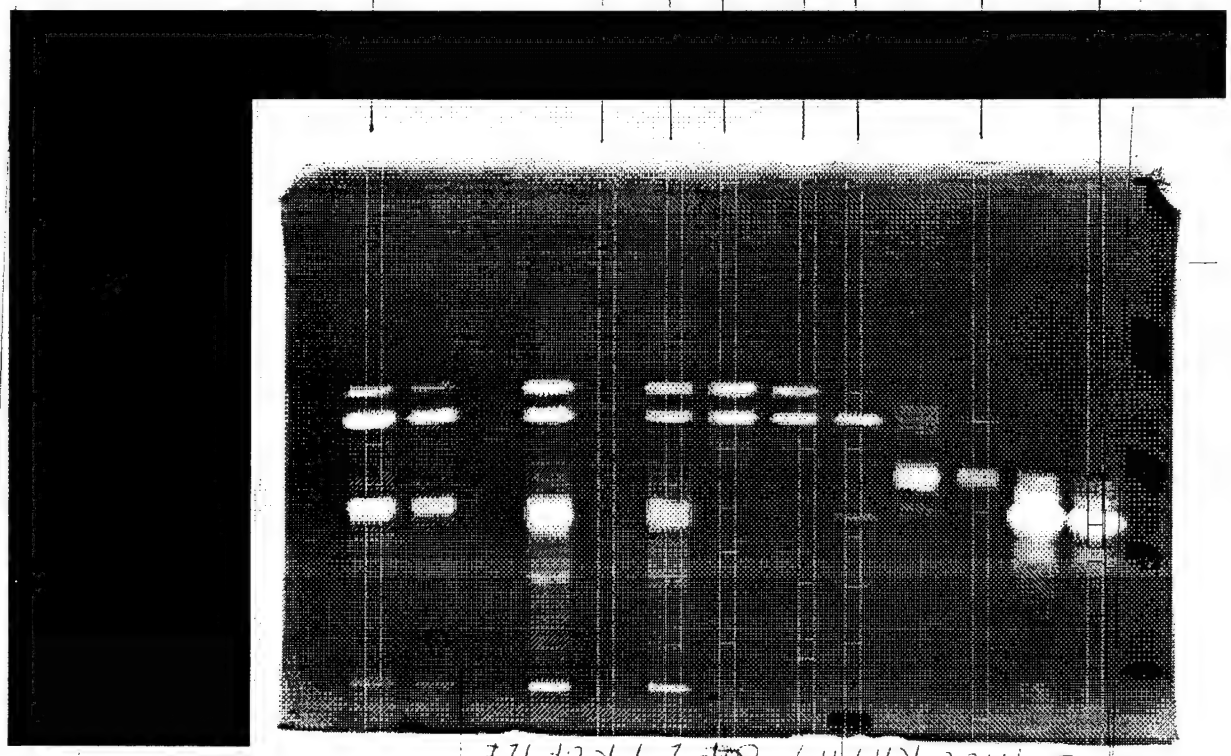
BENIGN

BENIGN NORMAL

MMP-9 ACTIVE

MMP-9 PRO

PEAK TRACK PROFILE

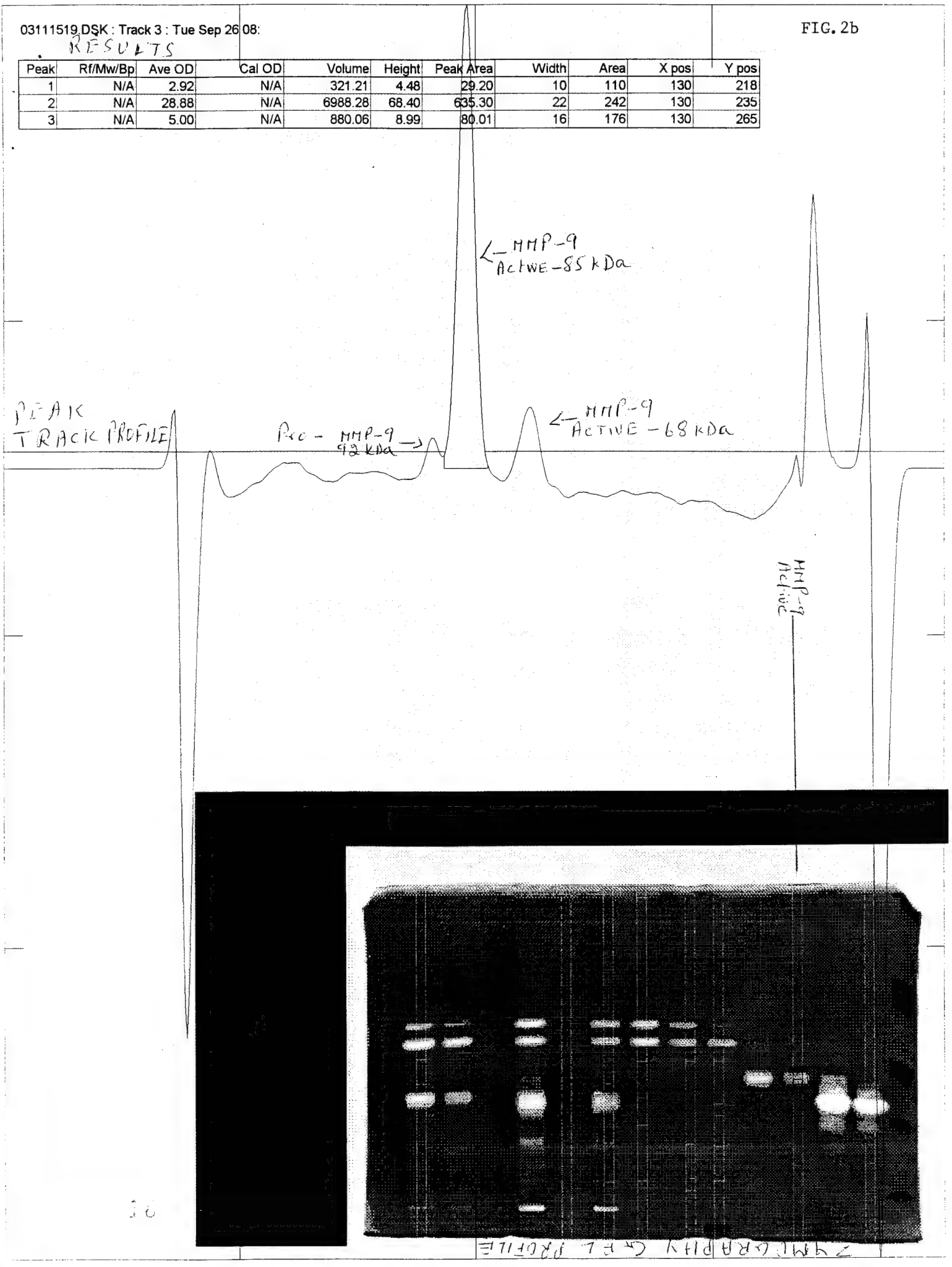


03111519.DSK : Track 3 : Tue Sep 26 08:

FIG. 2b

RESULTS

Peak	Rf/Mw/Bp	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos
1	N/A	2.92	N/A	321.21	4.48	29.20	10	110	130	218
2	N/A	28.88	N/A	6988.28	68.40	635.30	22	242	130	235
3	N/A	5.00	N/A	880.06	8.99	80.01	16	176	130	265



Figs. 3 - 4 show the profiles of normal breast tissue extracts (**Fig. 3a**), benign breast tissue extracts (**Fig.3b**) and the breast carcinoma tissue extracts (**Fig. 4a & b**). Quantitation of MMPs by zymography with the use of the GelBase software will facilitate the analysis of a large of samples. The activities estimated with the the above procedure shows 85% of the activity quantitated by substrate assays. Quantitation by substrate assays require lengthy procedures of molecular seive column chromatography for the separation of enzymes and their inhibitors.

Presence of tissue inhibitors in tissue extracts prevent the enzyme analysis directly from the tissue extracts as the activated enzymes bind directly to TIMPs and hence render them inactive. TIMPs can be destroyed by reduction and alkylation of tissue extracts without excessive destruction of the MMPs [29]. However, in the case of breast tissue extracts, TIMPs destruction was not complete as detected by reverse zymography.(results not shown) of reduced and alkylated extracts. Substrate assays require lengthy separation procedures and larger amounts of extracts.

SDS-PAGE substrate (zymography) analysis of extracts separates the proteins by the molecular weights and proteins and enzymes can be quantitated in µl quantities (**Fig. 1**).

The identity of each MMP secreted in breast tissue was further characterized using ACA54 molecular seive column. The fractions so obtained were scanned for enzyme bands by gelatin substrate zymography. The aliquots of fractions for each peak observed were then combined, reanalyzed by zymography. Peak sample from each gelatinases (92 kDa) and (72 kDa) were treated with APMA and the activated products analyzed by zymography. Results are presented in **Fig. 10** .This study shows that the enzymes found in breast tissues are gelatinases (metalloproteinases). Mrs of activated enzyme products were 84 kDa, 64 kDa for proenzyme 92 kDa and Mrs 62 kDa and faint bands at 45 kDa for proenzyme 72 kDa. The enzyme bands were inhibited by EDTA and 1,10 phenophthrailein (results not shown) by zymographic and substrate assay analysis.

Substrate (tritiated collgen) activity results for collagenase (MMP-1, interstitial collagenase,) and substrate (tritiated gelatin) for gelatinase A & B (MMP-9 + MMP-2) are presented in **Table 1**. Protein mg/g tissue and the wet weight of tissue analyzed is also reported in **Table 1**. The protein mg /gtissue was not significantly different in the different breast tissues. Carcinoma breast tissues show (**Table 1**)high gelatinase A and B activities (**98 units**) as compared to normal (**4 units**) and benign (**16.5 units**) tissues. **98 units** represent about **5 µg** of enzyme/g tissue. Table 1 alsoshows that collagenase (MMP-1) is **30 times** higher in IDC, breast carcinoma tissue (**5.5 units**) as compared to normal (**0.15 units**) tissue and 10 times higher than in benign tissue (**0.58units**). According to the specific activity of MMP-1, IDC tissue contains **one µg** of MMP-1/g tissue. This is enough to digest the collagen and together with high gelatinases, these enzymes can disrupt the ECM

03111519.DSK : Track 4 : Tue Sep 26 08:

RESULTS

FIG. 3a

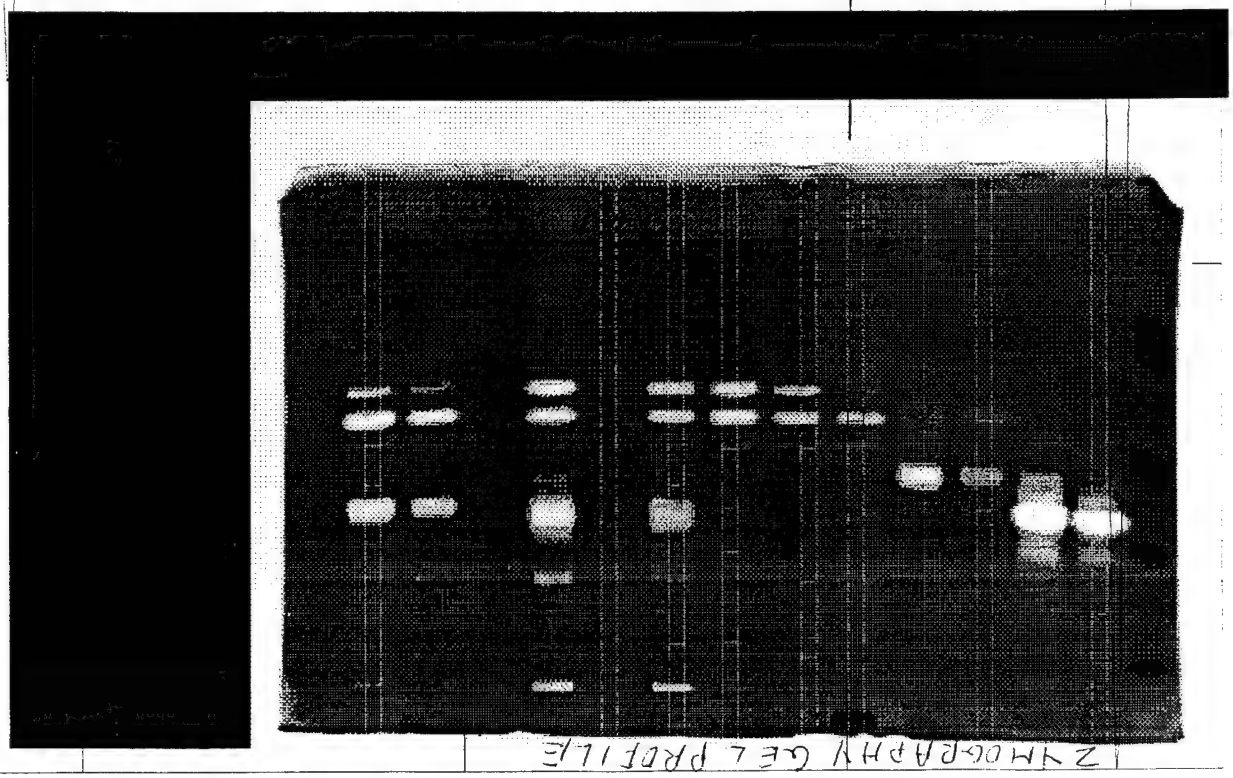
Peak	Rf/Mw/Bp	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos
1	N/A	31.21	N/A	5492.92	74.32	499.36	16	176	197	265
2	N/A	2.48	N/A	163.93	3.55	14.90	6	66	197	280

← MMP-2 PROENZYME

← MMP-2 LATENT

PEAK TRACK PROFILE

NORMAL BREAST TISSUE EXTRACT



ZYMOGRAPHY GEL PROFILE

20

03111519.DSK : Track 5 : Tue Sep 26 08:

FIG. 3b

RESULTS

Peak	Rf/Mw/Bp	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos
1	N/A	29.55	N/A	5200.68	69.08	472.79	16	176	221	265
2	N/A	29.07	N/A	4157.53	58.64	377.96	13	143	221	280

← MMP-2 LATENT (PRO)

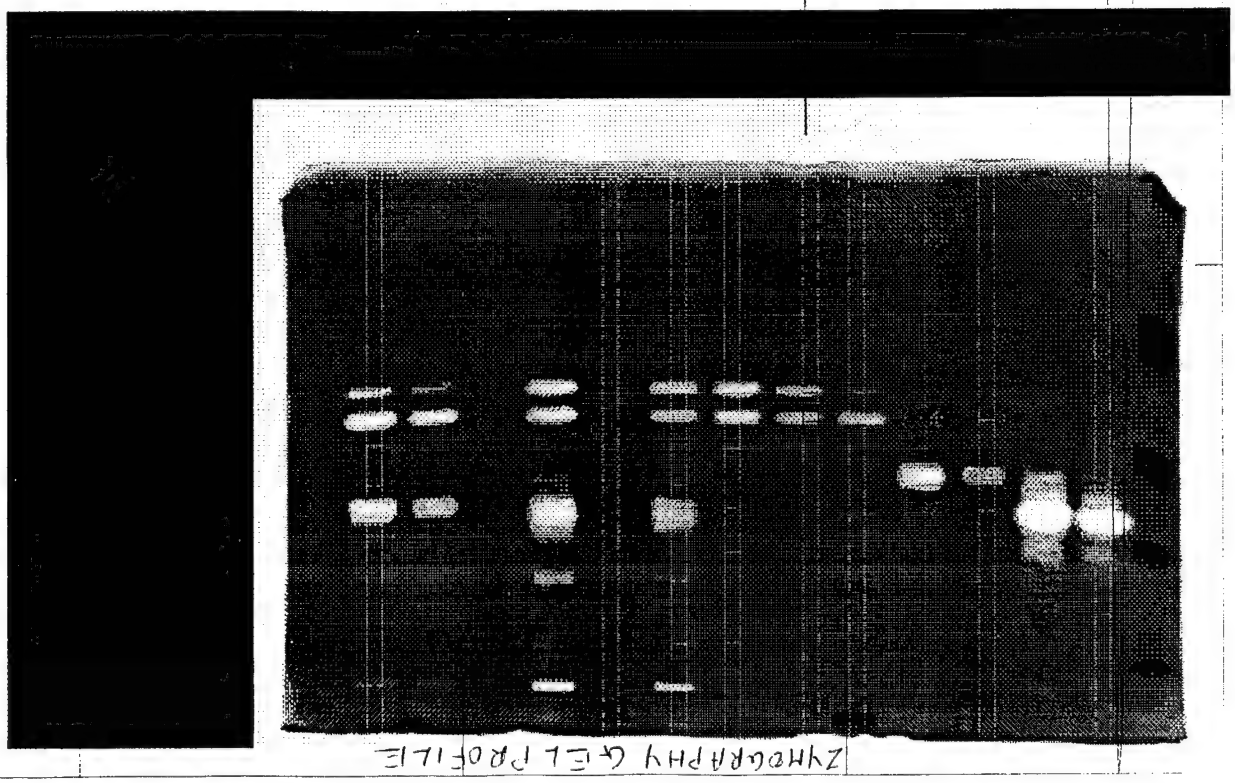
← MMP-2 ACTIVE

MMP-2 → LATENT (PRO)

← MMP-2 ACTIVE

TRACK PROFILE

BENIGN BREAST TISSUE EXTRACT



21

03111519.DSK : Track 7 : Tue Sep 26 08:

FIG. 4a

• RESULTS –

Peak	Rf/Mw/Bp	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos	
1	N/A	13.21	N/A	2616.23	35.46	237.84	18	198	290	125	← ~200 KDa
2	N/A	6.24	N/A	1510.24	8.08	137.29	22	242	290	149	
3	N/A	10.93	N/A	2403.69	17.92	218.52	20	220	290	182	← ~140 KDa
4	N/A	36.59	N/A	16097.69	94.52	1463.43	40	440	290	215	← MMP-9
5	N/A	5.38	N/A	1125.14	10.74	102.29	19	209	290	232	← MMP-9 Act
6	N/A	29.06	N/A	5433.51	68.96	493.96	17	187	290	267	← MMP-2
7	N/A	36.22	N/A	5578.49	75.11	507.14	14	154	290	281	← MMP-2 Act

MMP-9 →

MMP2 → LAT

← MMP-2 Act

← 200KDa Aggregate MMP-9

Aggregate → MMP-2

TRACK PROFILE PEAKS

BREAST CARCINOMA II TISSUE EXTRACT

ZYMOGRAPHY GEL PROFILE

03111519.DSK : Track 8 : Tue Sep 26 08:

RESULTS —

FIG. 4b

Peak	Rf/Mw/Bp	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos	
1	N/A	5.26	N/A	1214.39	13.60	110.40	21	231	448	128	<—
2	N/A	37.76	N/A	16615.71	137.51	1510.52	40	440	448	219	<— MMP-9 LAT
3	N/A	6.22	N/A	1094.44	11.27	99.49	16	176	448	232	<— MMP-9 ACT
4	N/A	2.99	N/A	328.93	4.72	29.90	10	110	448	253	
5	N/A	60.16	N/A	12572.96	146.68	1143.00	19	209	448	265	<— MMP-2 LAT
6	N/A	25.71	N/A	3110.80	46.03	282.80	11	121	448	279	<— MMP-2 ACT

MMP-9 →

<— MMP-2 LATENT

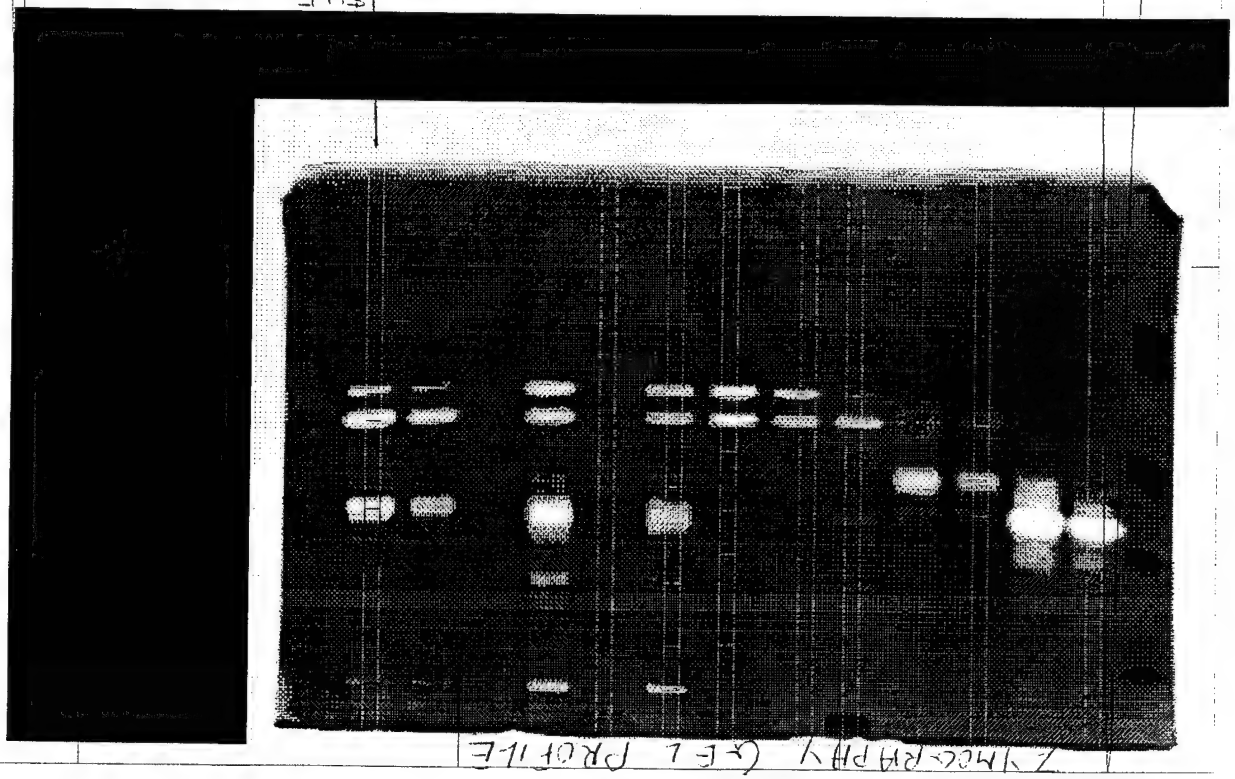
<— MMP-2 ACTIVE

MMP-9 ACT

PEAKS TRACK PROFILE

<— ~200 KDA AGGREGATES

BREAST CARCINOMA TISSUE II EXTRACT



GEL PROFILE (ZYMOGRAPHY)

and allow the tumor cells to escape. No stromelysin-1 was found in any extracts. Stromelysin-3 cannot be estimated by protein substrate assay and was not quantitated. No metallo-elastase or PUMP-1 (matrilysin) was detected in breast tissue. Several bands of serine proteases were observed in carboxymethylated transferrin zymography (result not shown). No attempt was made to characterize or quantitate them. This line of research may be pursued in later years.

6.B.2. Immunohistochemical evaluations of breast tissue:

The results are presented in **Figs. 5- 8** . The parrafin sections are stained with anti-TIMP-1 -MMP-9 and -MMP-2 IgGs, followed by IgG-peroxidase as reported in Methods section. Immunoperoxidase staining reveals brown granules at the site of antigen-antibody binding. The breast tissues were stained with only three antibodies -Anti-TIMP-1 (whole molecule), -MMP-9 (MBR, metal binding region peptides) and -MMP-2 (MBR, metal binding region peptide). We hope to stain the same tissues with other MMP and TIMP antibodies. **Fig. 5a** shows the routine H&E (hematoxylin and eosin, histopathology stain) staining of benign breast tissue and exhibiting pink colored sytoplasm and purple nuclei. Epithelial to stroma ratio (E:S) was 1:10. Results of staining benign tissue with anti-TIMP-1 or anti-MMP-9 or anti-MMP-2 are presented in in **Fig. 5b, 5c,and 5d** respectively. Weak staining of epithelium and macrophages only; stroma (connective tissue component of benign and malignant tumors) is negative. There was negative staining of stroma and epithelial but some staining of macrophages with anti-MMP-9 IgGs (**Fig. 5c**). On the other hand, no staining of epithelial but staining of stroma was found with anti-MMP-2 (**Fig. 5d**).

Fig. 6 a, b, c & d show staining of fibrocystic disease (benign epithelial hyperplasia) breast tissue with anti-TIMP, -MMP9 and -MMP-2 No staining of stroma or epithelium with anti-TIMP-1 IgG. As in Fig. 5, negative staining of stroma and weak stainig with epithelium with anti-MMP-9 IgG (**Fig. 6c**).Anti-MMP-2 (**Fig. 6d**) stained stroma and negative staining of epithelium.

Fig. 7 & 8 show staining of breast carcinoma tissues (IDC, infiltrating ductal carcinoma). **Fig. 7a & 7a** show routine H & E staining for stroma and tumor cells. Anti-TIMP-1 stained weakly positive (**Fig. 7b**) for tumor cells in one IDC tissue but negative staining for second IDC tissue (Fig. 8b). Stroma was negative for both the tIDC tissues. The important finding is presented in **Figs. 7c & 8** confirming the presence of MMP-9 in the IDC tissues as seen by zymography of tissue extracts. There is an intense staining of tumor cells with anti-MMP-0 IgG in both breast cancer tissues. There is weak staining of tumor cells and stroma with anti-MMP-2 IgG. Zymography (**Fig. 1**) and substrate activity results (**Table 1**) show high gelatinase activity of 98 units/g tissue (IDC) and is statistically significant. This is confirmed by high staining of MMP-9 by immunohistochemical analysis.

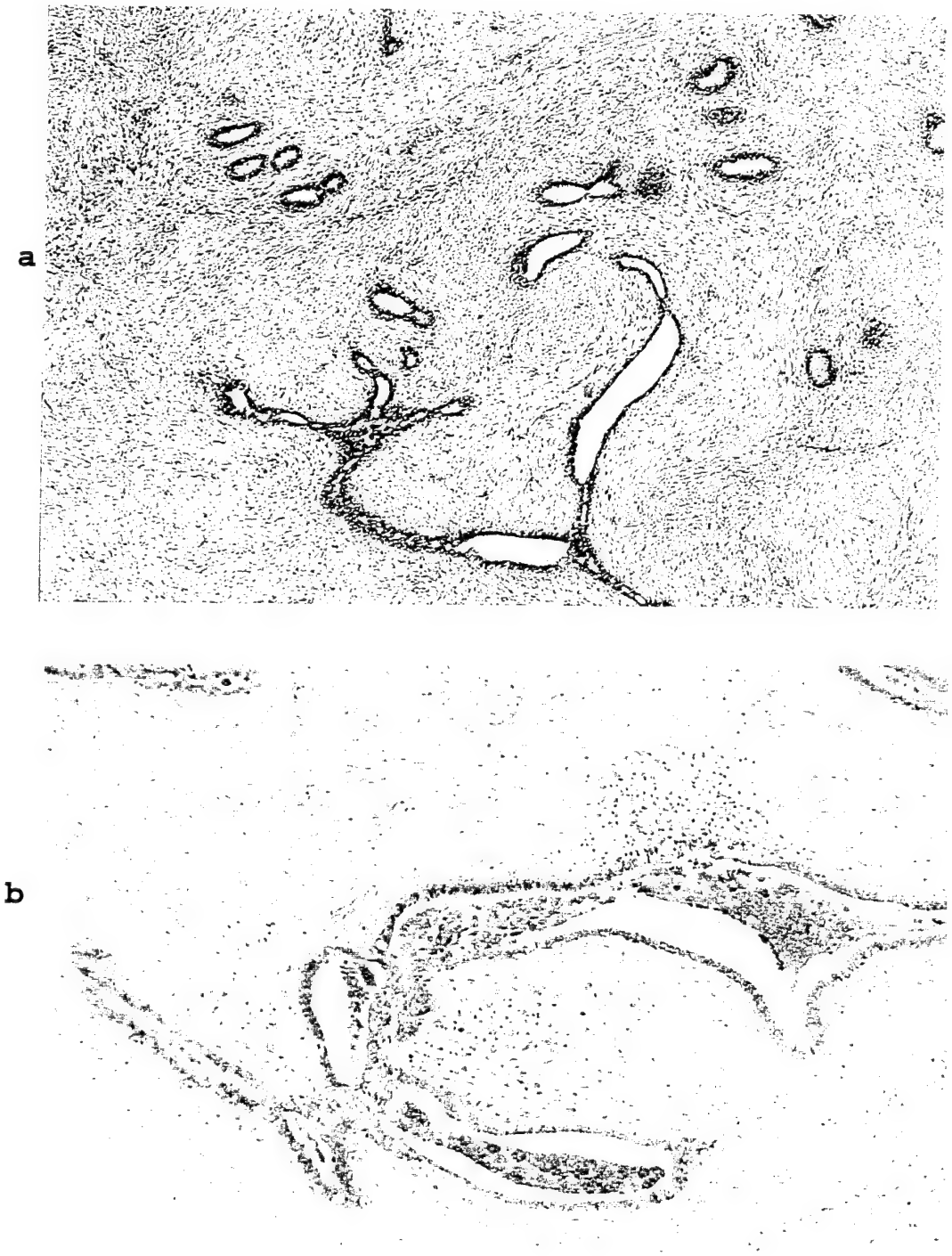


FIG. 5 Immunohistochemical staining of breast tissue (fibroadenoma, benign tumor). **a:** H&E staining. **b:** Immunoperoxidase staining with anti-**TIMP-1** IgG.showing weak staining of epithelium and macrophages; stroma is negative.Epithelial to stroma ratio (E:S) = 1:10

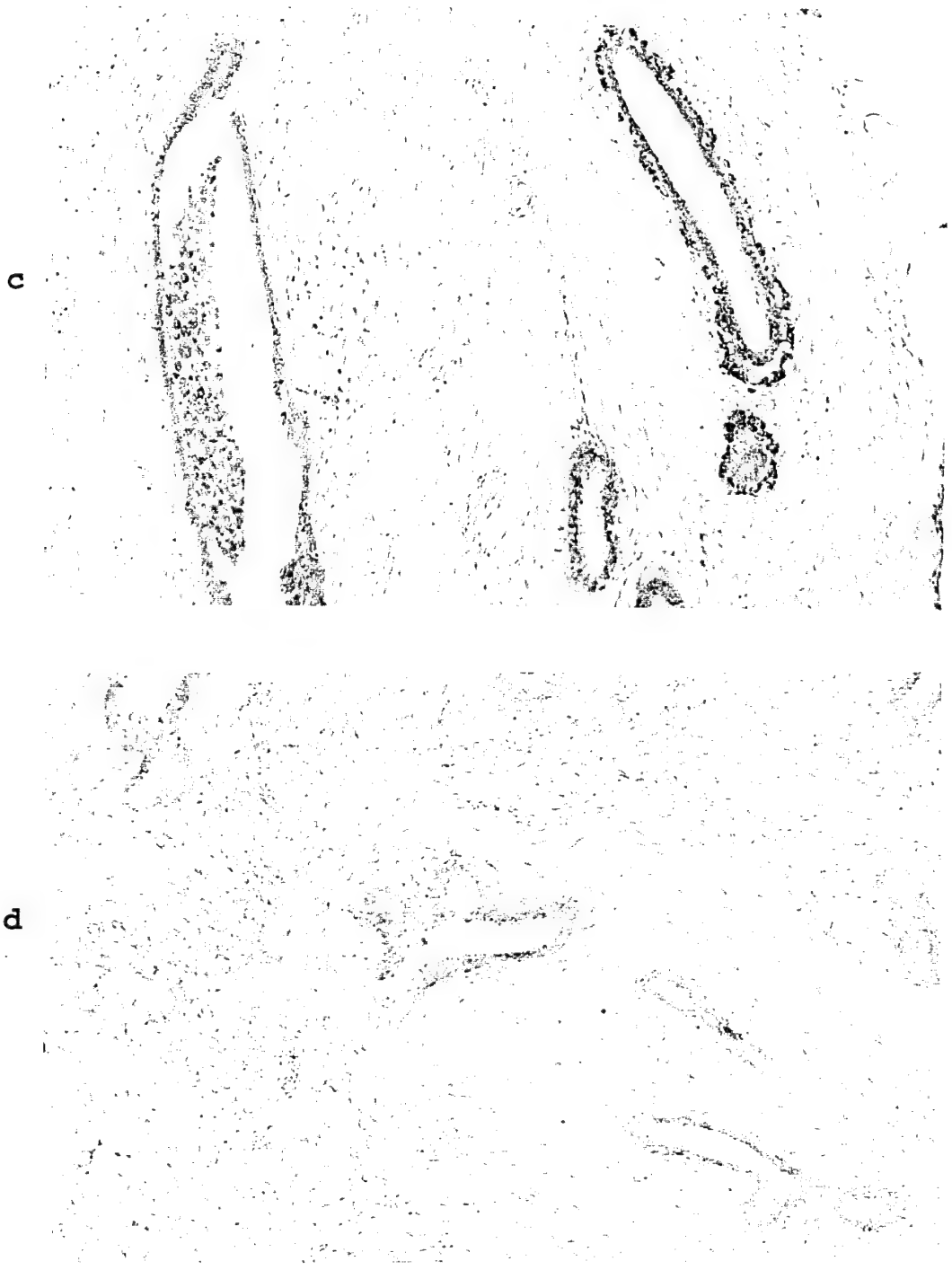
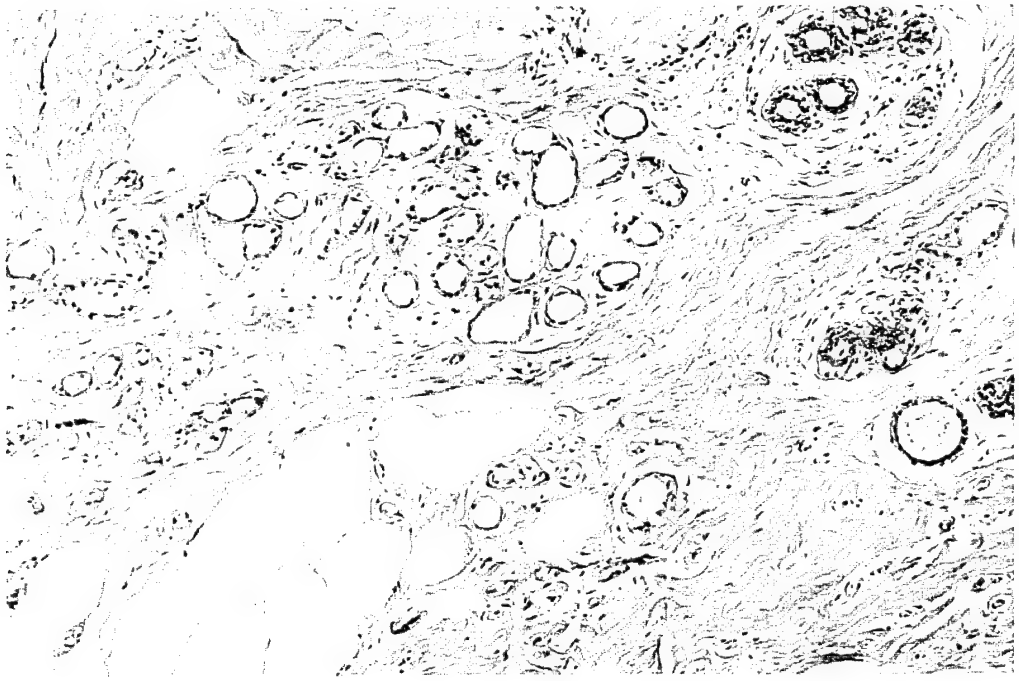


FIG. 5 Immunohistochemical staining of breast tissue (fibroadenoma, benign tumor). **c:** Immunoperoxidase staining with anti-**MMP-9 (92 kDa gelatinase) IgG**, negative staining of epithelial and stroma; staining of macrophages. **d:** Immunoperoxidase staining with anti-**MMP-2 (72 kDa gelatinase)** IgG, negative staining of epithelium; positive staining of stroma. (E:S) = 1:10.

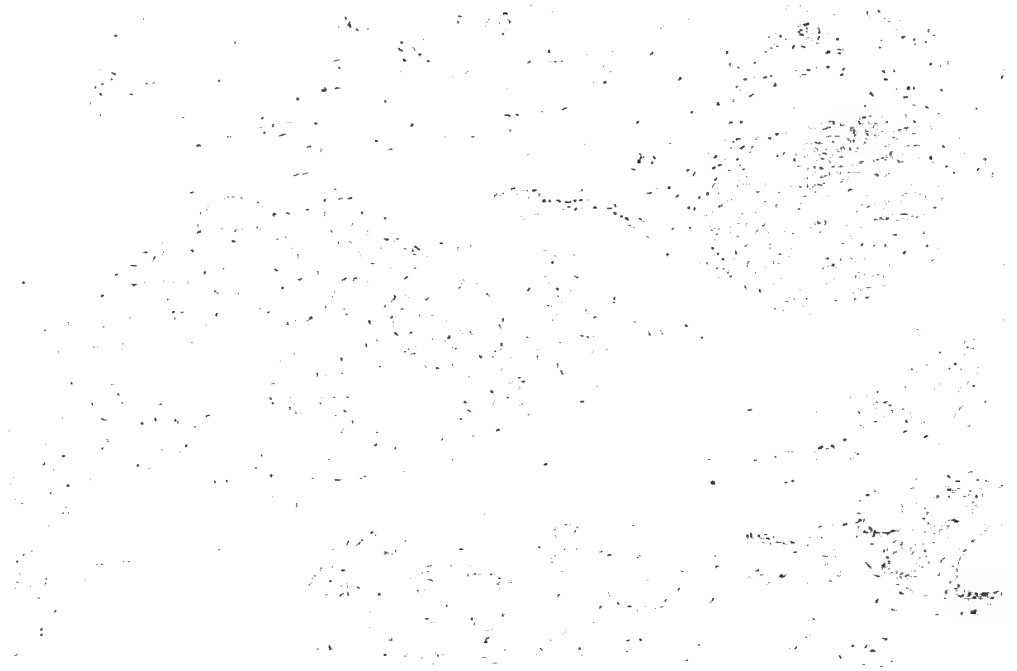
a



b

FIG. 6 Immunohistochemical staining of breast tissue (fibrocystic disease , benign epithelial hyperplasia, area away from adenoma tumor). **a:** H&E staining of the tissue. **b:** Immunoperoxidase staining with anti-**TIMP-1** IgG showing negative reaction for epithelium and stroma. (E:S) = 1:5.

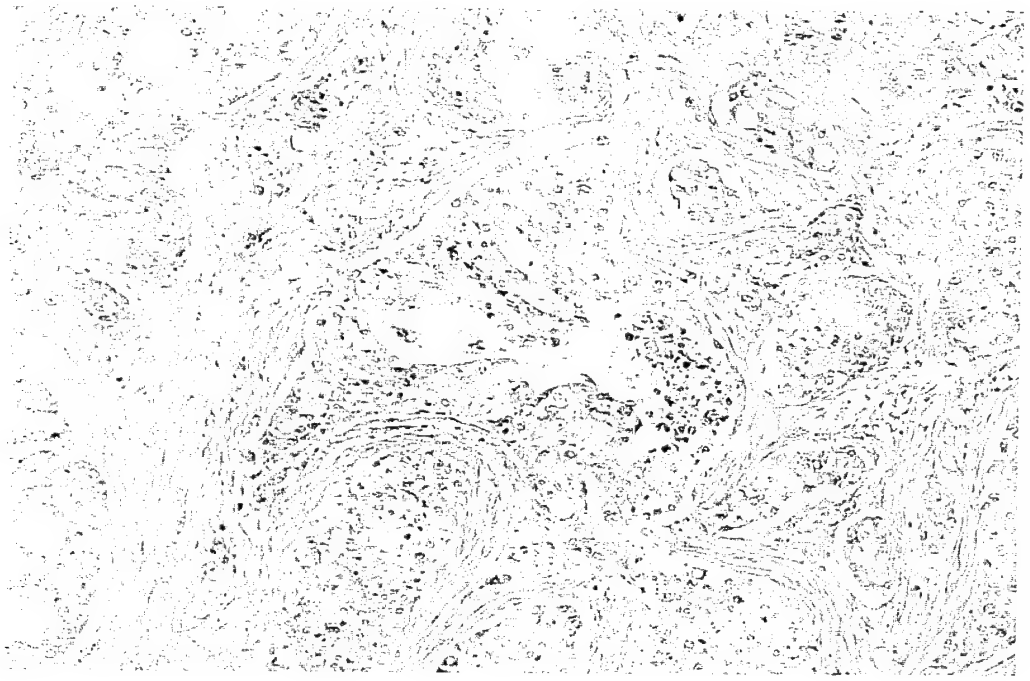
c



d

FIG. 6 Immunohistochemical staining of breast tissue (fibrocystic disease , benign epithelial hyperplasia, area away from adenoma tumor). **c:**Immunoperoxidase with anti-**MMP-9 (92 kDa gelatinase) IgG**, negative staining of stroma and weak staining of epithelial. **d:** Immunoperoxidase staining with anti-**MMP-2 (72 kDa gelatinase)** IgG, negative staining of epithelium; positive staining of stroma. (E:S) = 1:5.

a



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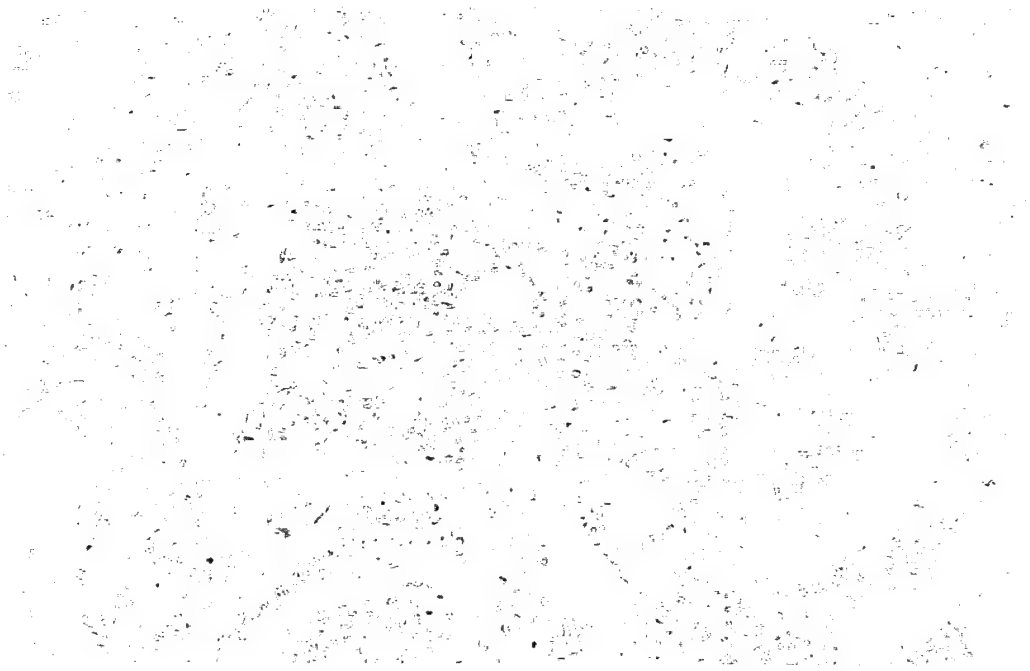
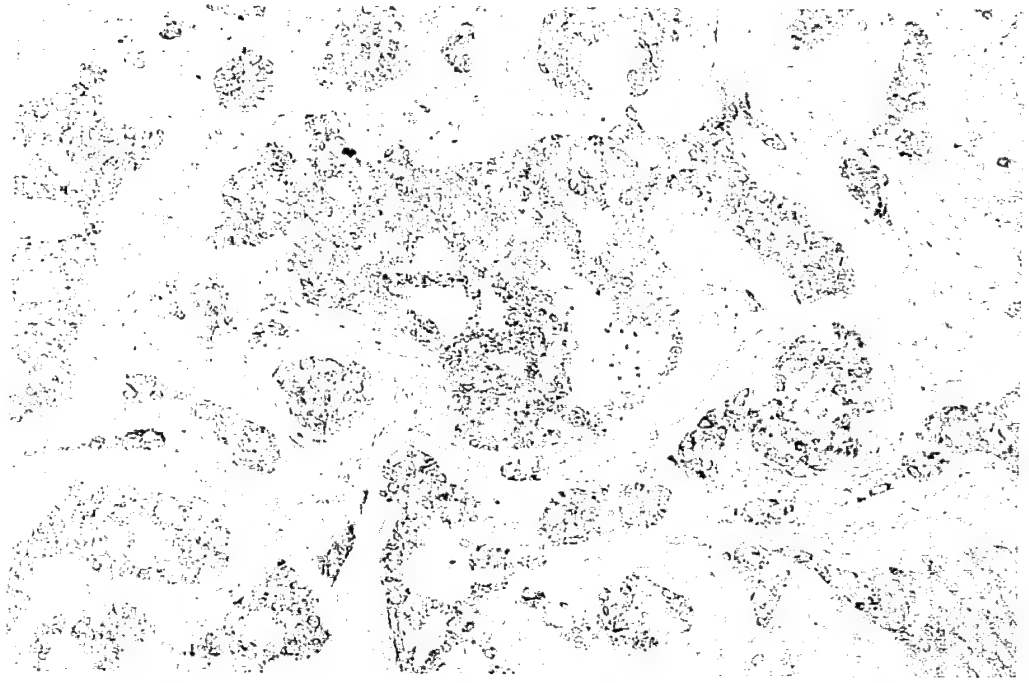


FIG. 7 Immunohistochemical staining of breast tissue (infiltrating ductal carcinoma [IDC]). **a:** H&E staining of the tissue. **b:** Immunoperoxidase staining with anti-**TIMP-1** IgG showing weak staining of tumor cells; stroma is negative. (E:S) = 4:1.

c



d

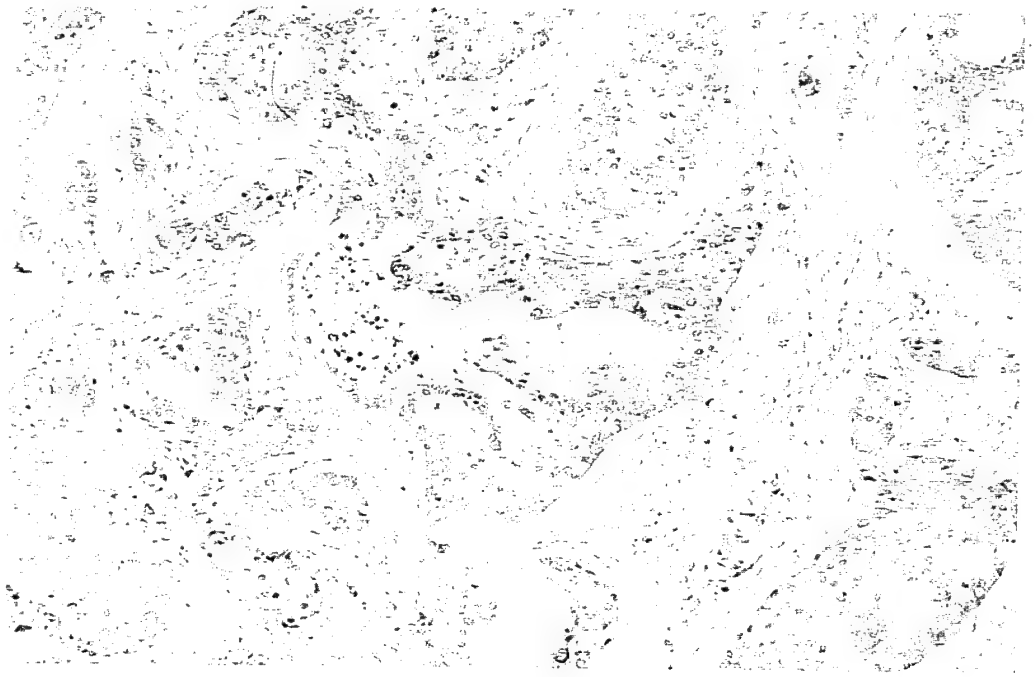
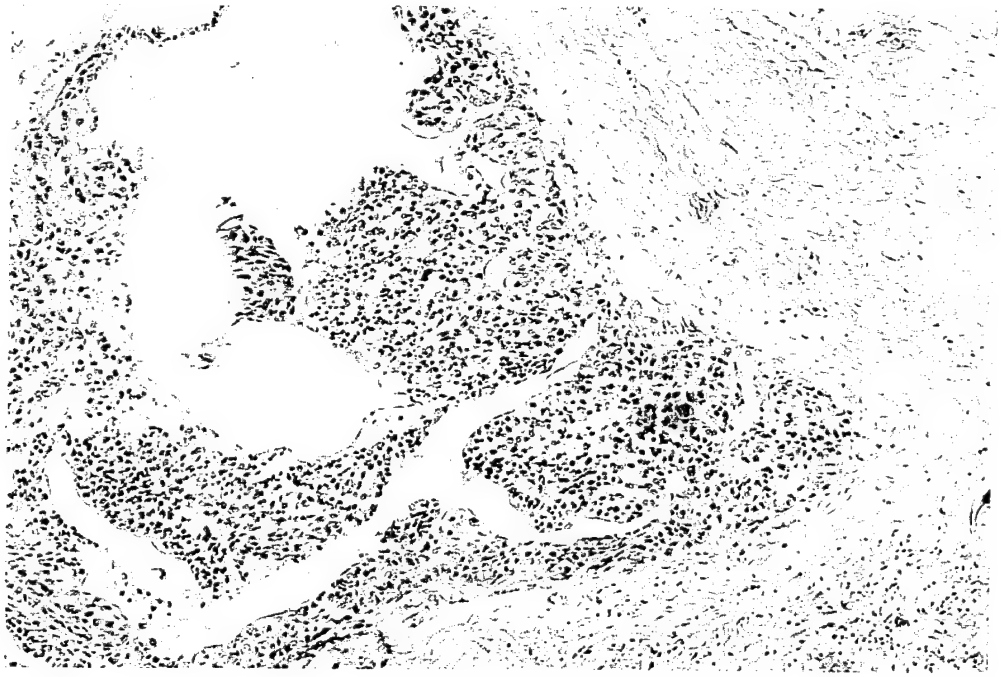


FIG. 7 Immunohistochemical staining of breast tissue (infiltrating ductal carcinoma [IDC]). **c:** Immunoperoxidase staining with anti-**MMP-9 (92 kDa gelatinase)** IgG, negative staining of stroma and strong staining of tumor cells **d:** Immunoperoxidase staining with anti-**MMP-2 (72 kDa gelatinase)** IgG, weak staining of tumor and stroma. (E:S) = 4:1.

a



b

FIG. 8 Immunohistochemical staining of breast tissue (intraductal carcinoma). **a:** H&E staining of the tissue. **b:** Immunoperoxidase staining with anti-**TIMP-1** IgG showing negative staining of tumor cells; stroma is negative. (E:S) = 4:1.

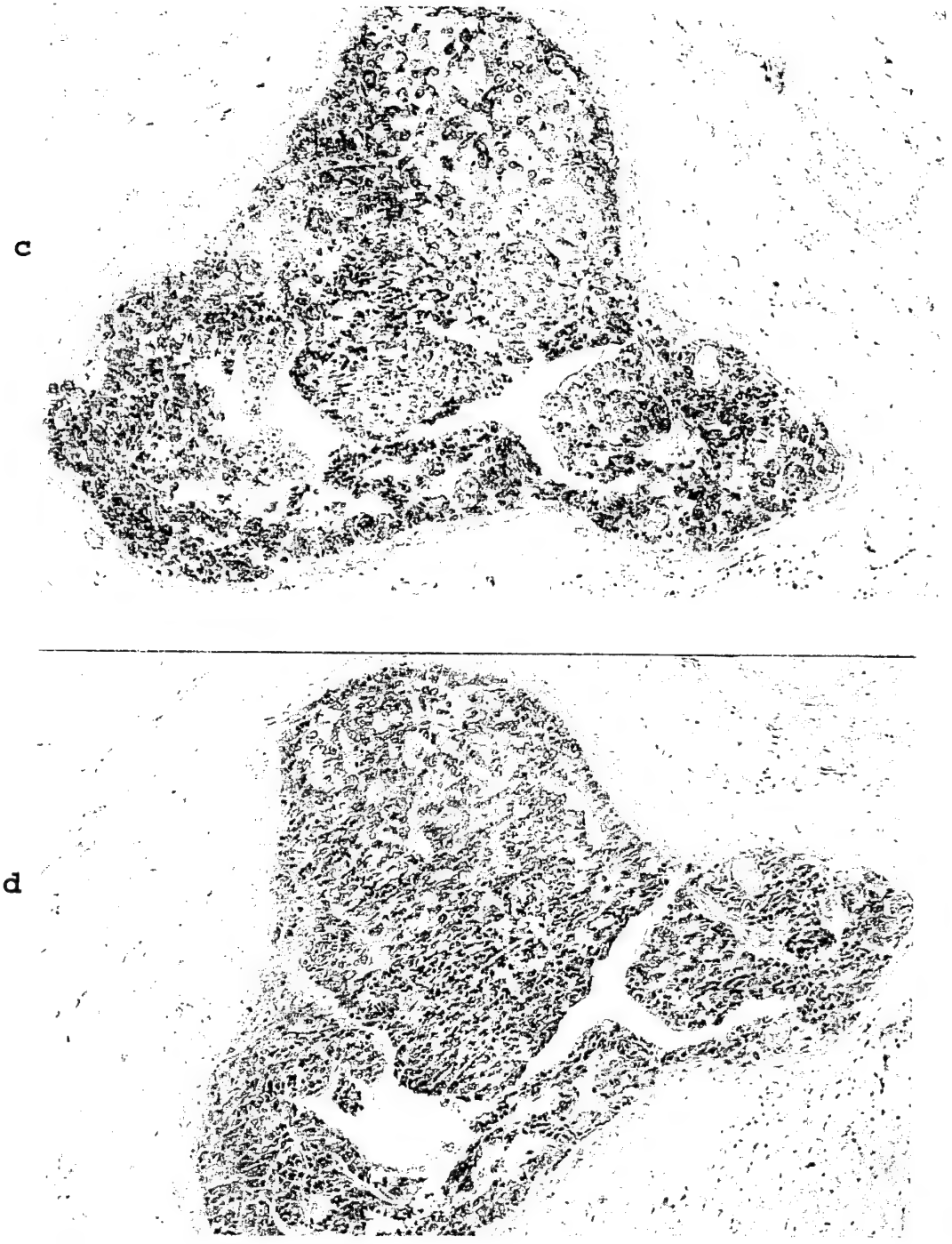


FIG. 8 Immunohistochemical staining of breast tissue (intraductal carcinoma). **c:** Immunoperoxidase staining with anti-**MMP-9 (92 kDa gelatinase)** IgG, negative staining of stroma and strong staining of tumor cells
d: Immunoperoxidase staining with anti-**MMP-2 (72 kDa gelatinase)** IgG, staining of tumor and stroma.
(E:S) = 4:1.

6.B.3. Analysis of TIMPs in breast tissue:

The breast tissue extracts were scanned for TIMPs by reverse zymography. TIMP-1, -2 & -3 are detected in ng quantities. The tissue extracts are fractionated by SDS-PAGE (12,5% acrylamide, gelatin and enzyme media) electophoresis The uncleared blue bands are due to the TIMPs present in the extracts or conditioned media. The results of breast tissue extracts are reported in **Fig. 9**. All 3 TIMPs are present in different amounts in the breast tissue extracts Only 1M GuHCl extracts can be scanned for TIMPs. The Triton extracts contain other soluble proteins and it becomes difficult to distinguish the TIMP bands from the regular same molecular weight protein bands that did not diffuse out of gel during incubation. We hope to use this method quantitatively by scanning the bands by the GelBase software.

6.B.4. Analysis of secreted MMPs and TIMPs in various tumor cell lines:
Several cell lines were scanned for secreted MMPs and TIMPs and the results are reported in **Fig.11 & 12** respectively. The cell lines used were MCF-7, DA-3, 101A-HMT, PPC-1, PC-3 and MDA-MB. All 0% serum free conditioned media in resting phase had to be concentrated five times to reveal cleared bands of enzymes. Stimulation of cell lines by PMA (50 ng/ml) in 0% serum free media showed 20-500 times enzyme bands The results ar reported in **Fig 11**.. MCF-7 secretes mainly MMP-9 with and without PMA stimulation. No MMP-2 is found even in the PMA stimulated media. These results are very interesting in the light that confocal studies reported below show a big band of 72 kDa in the transmembrane area.

Another important information (**Fig. 13**) we obtained with PMA stimulated MCF-7 cells was that addition of anti-CD44v-III IgG (1µg/ml media) activated the secreted 92 kDa MMP to active fragments of 84 kDa, 63 kDa and 48 kDa enzyme (**lane 2**). Rat anti-CD44 (standard form) IgG (**lane 1**) did not activate the secreted MMP-9 Anti-CD44v-VI (**lane 3**) showed some activation. The cells were in cotact with PMA and the antibodies for one hour and four hours respectively. The media is removed, filtered and analyzed by gelatin zymography. These results are very exciting and hope to expand these studies into second year of the granting period. These findings may relate to the calcium channeling inside the cell.

Reverse zymography results of the conditioned cell line media are reported in **Fig. 12** MCF-7 and PPC-1 secrete vast amounts of TIMP-1 TIMP-3 binds to cell matrix and dishes have to be extracted with either 1M GuHCl or 0.1% SDS to quantitate by reverse zymography and hence the visualiztion of TIMP-3 is poor and only overly expressed and nonbinding TIMP-3 can be seen in PMA stimulated MCF-7 media. The TIMP experiments have to be refined to obtain meaningful data.

6.B.5. Immunofluorescense staining and confocal microscope analyses:
Human breast cells e.g. MCF-7 were incubated with fluorescin-labeled

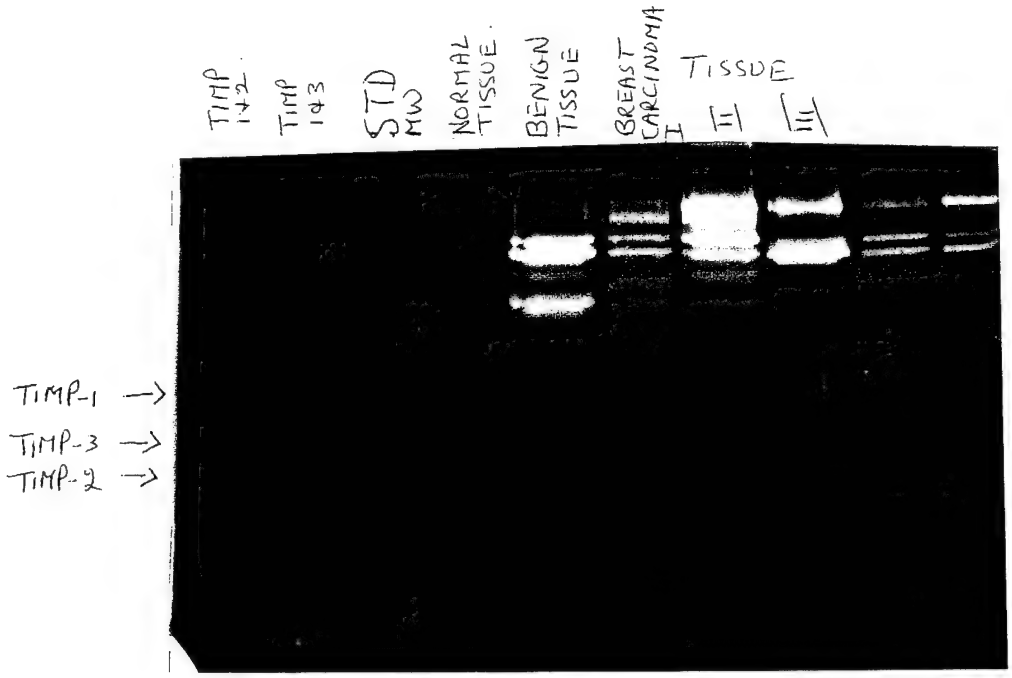


FIG. 9. Reverse zymography of breast tissue extracts (1M GuHCl) showing presence or absence of TIMPs-1, -2 or -3. This method does not quantitate the TIMPs.

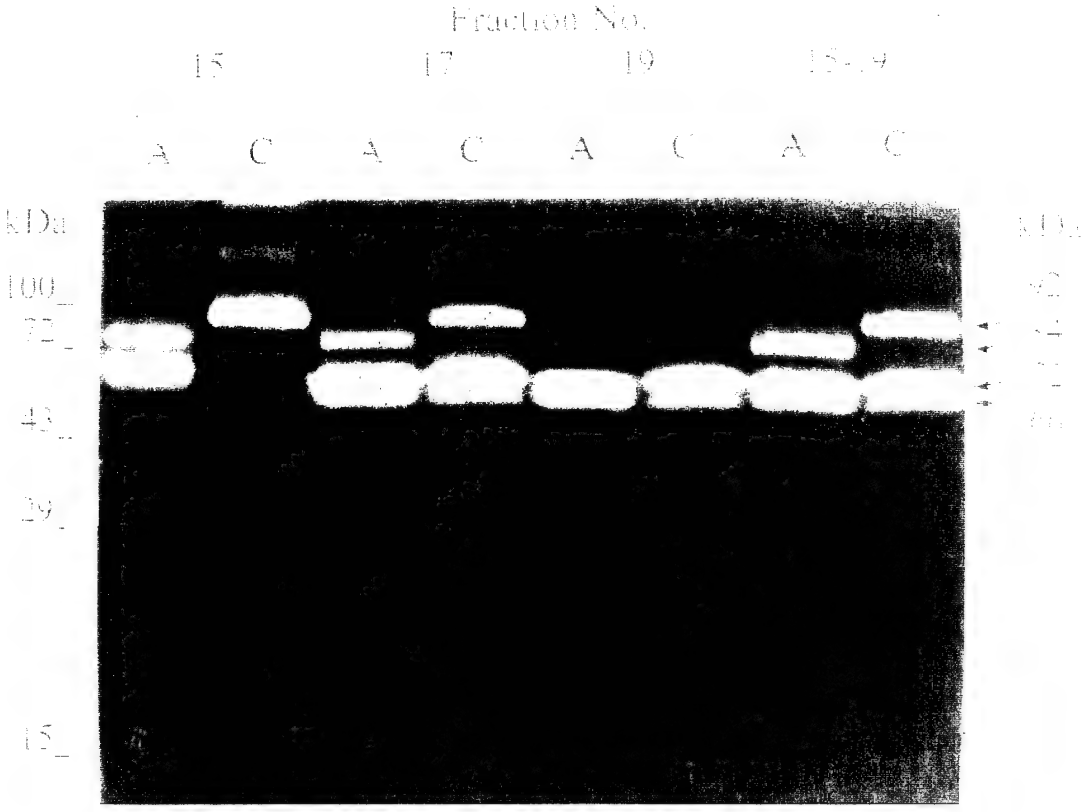


FIG. 10. Zymography (gelatin) of molecular seive column fractions of breast tissue extracts (1M GuHCl or Triton X-100) with or without APMA (mercurial salt) activatable forms of gelatinases (MMP-9 & MMP-2). Fraction # 15 shows mainly MMP-9 (92 kDa gelatinase); fraction # 19, mainly MMP-2 (72 kDa gelatinase); fraction # 17, mixture of MMP-9 & MMP-2.

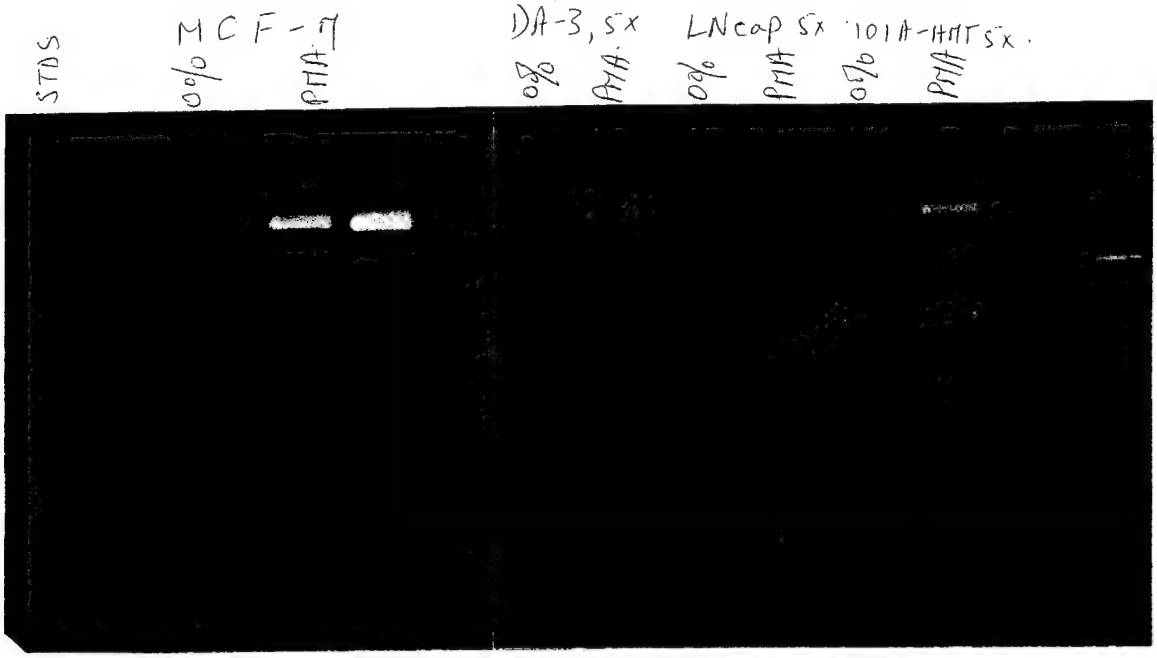


FIG. 11. Zymographic (gelatin) evaluation of conditioned serum free media of various tumor cell lines. **MCF-7**, breast tumor cell line; **DA-3**, a murine tumor cell line; Lncap, prostatic tumor cell line and 101A-**HMT**, a human tumor cell line causing breast and lung tumors in mice (a gift from Goodwin Inst. , Florida).

0% = no serum in media
PMA = Phorbol ester (50 ng/ml) in 0% media.

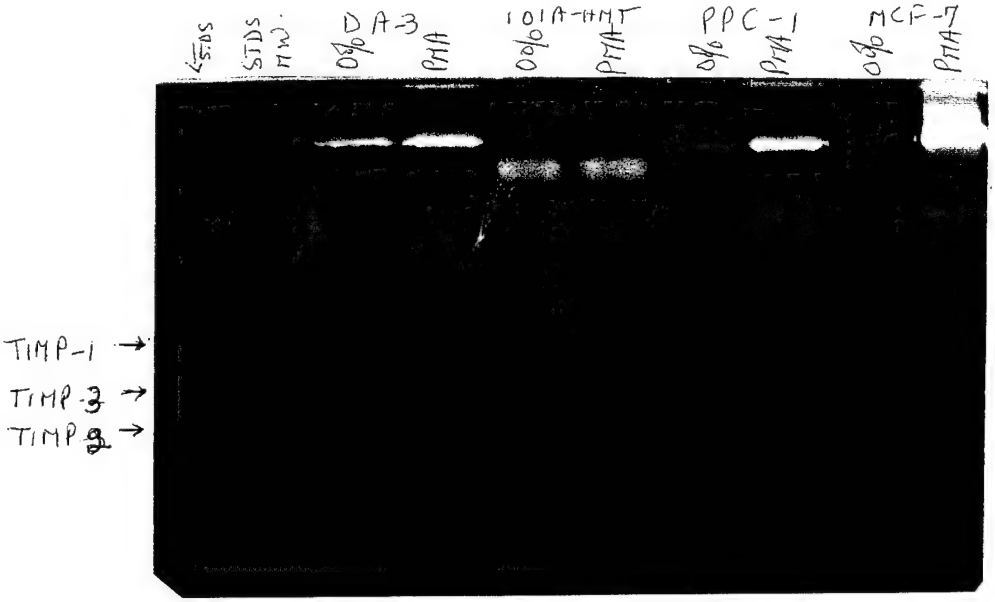


FIG. 12. Reverse zymography (gelatin) of conditioned serum free media of various tumor cell lines. **MCF-7**, breast tumor cell line; **DA-3**, a murine tumor cell line; **PPC-1**, a cloned tumor line from PC-3,a prostatic tumor cell line; and **101A-HMT**, a human tumor cell line causing breast and lung tumors in mice (a gift from Goodwin Inst. ,Florida).

0% = no serum in media
PMA = Phorbol ester (50 ng/ml) in 0% media.

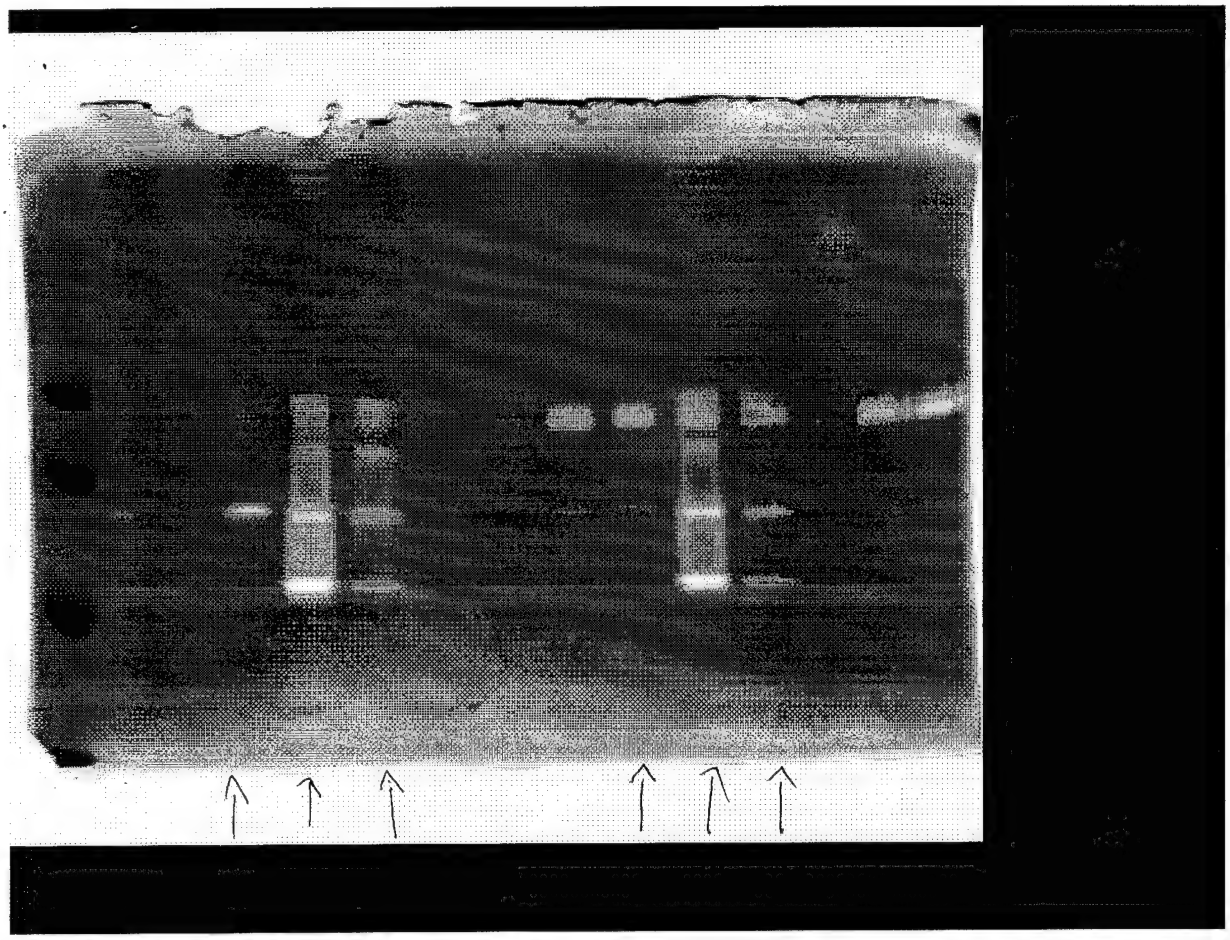


FIG. 13 Zymographic (gelatin) detection of secreted MMPs from serum free (0%) conditioned media of **MCF-7** breast tumor cell line. The cells were treated with PMA (50 ng/ml) together with 1) rat anti-CD44 (standard form of CD44); 2) rabbit anti-CD44v-III (exon inserted between exons 5 & 15, [ ]) and 3) rabbit anti-CD44v-VI (exon-14-CD44) for one and four hour. The media was removed and analyzed for MMP profile. There is activation of MMP-9 observed in media treated with anti-CD44-v-III and CD44-v-VI IgGs. No activation was observed with rat anti-CD44 (standard form) and only PMA treated media.

rat anti-CD44 antibody and rhodamine-labeled rabbit anti-MMP-9 or rabbit anti-MMP-2; cells were washed and labeled samples excited with a krypton-argon ion mixed-gas laser and examined with a laser scanning Confocal microscope (Multiprobe 2001 Invert CLSM System, Molecular Dynamics) using a 63 X-oil immersion and an imaging processing device. Cells were photographed with Kodak Tri-X-film. The results are presented in **Figs. 14-15**. MCF-7 cells used were in resting and PMA stimulated phase. **Fig. 14a** shows the staining (red color) with anti-MMP9 IgG of MCF-7 cells in resting phase. Even though MMP-9 is not secreted in sufficient amounts, MMP-9 is found in sufficient amounts in the cell near the membrane. In contrast, **Fig. 14b** shows that PMA-stimulated cells are full of MMP-9 stain towards the center of the cells. CD44 is found in transmembrane (green color).

Fig. 15a & b show the staining with anti-MMP-2 IgG in resting and stimulated phases. There is faint staining of MMP-2 (red color) along with CD44 (green color) in the edge of the cell around the membrane. In contrast, intense MMP-2 staining in the PMA-stimulated cells is observed along with CD44 (green color) in the transmembrane area. What is puzzling is that MMP-2 is not secreted by stimulated cells. Is MMP-2 bound to TIMP-2 wihich in turn is bound to MT-MMP [46]? This finding is also very exciting and future studies are needed to understand the underlying mechanism.

7. CONCLUSIONS:

MMPs and TIMPs represent a class of metalloproteases and their tissue inhibitors secreted by various types of cells, including epithelial, fibroblasts, and macrophages MMPs have been implicated in degradation of basement membrane during cancer invasion and metastasis Their activity is controlled, in part, by natural inhibitors (TIMPs). The imbalance created in the secretion of their tissue inhibitors (TIMPs) has been implicated by us for prostate cancer [26].The present study in its first year clearly demonstrates that 1) extraction of enzymes from the tissues and 2) immunohistochemical analysis, shows the presence of different levels of latent and active forms of MMPs in normal, begnin and cancer human breast tissues. The extraction of these tissues also show the presence of diferent types and levels of TIMPs by reverse zymography of tissue extracts. These findings strongly suggest that the basement membrane underlying breast epithelium probably undergoes rapid turnover due to matrix degrading enzymes secreted by various resident cells in the breast tissue.

A novel finding of this study is that gelatinase B (MMP-9) was found only in breast tumor tissue as demonstrated by gelatin zymography and immunohistochemical analysis of the same tissues. Other classes of MMPs such as MMP-1 (interstitial collagenase) and gelatinase A (MMP-2) were found in all classes of breast tissue. Normal and benign tissues showed the presence of only MMP-2 and MMP-1 in 10 to 30 times lower in amount

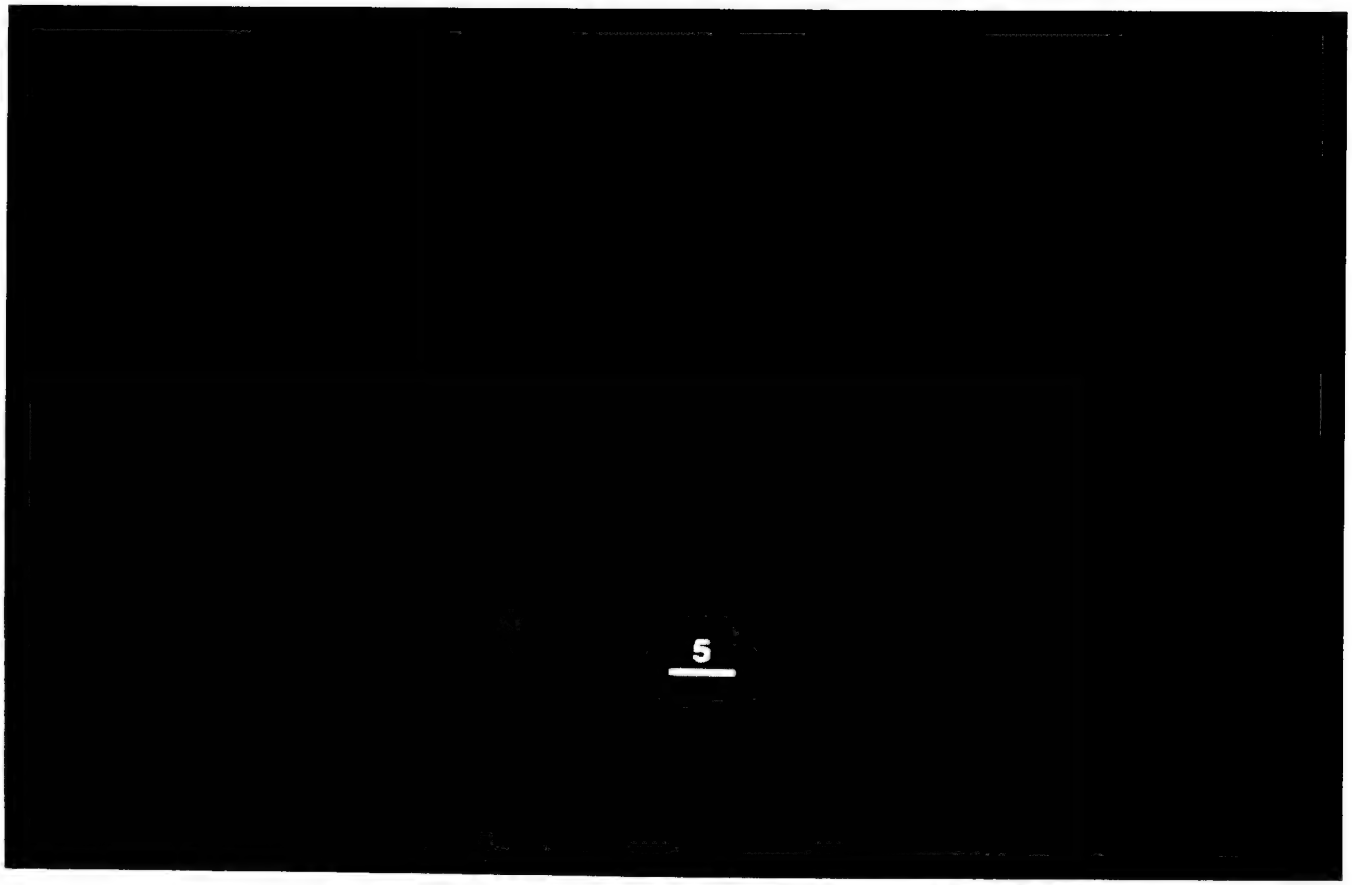


FIG. 14a Immunofluorescence staining of MMP-9 and of CD44 in MCF-7 breast cancer cell line using confocal microscope. The cells were in resting phase in serum free media. Rat anti-CD44 IgG was used, followed by fluorescein-labeled (green color) goat anti-rat IgG. Rabbit anti-MMP-9 IgG was used, followed by rhodamine-conjugated (red) goat against rabbit IgG. These analyses show that MMP-9 stains fairly well and is found near the cell surface and some in granules. CD44 stains weakly at the cell surface.

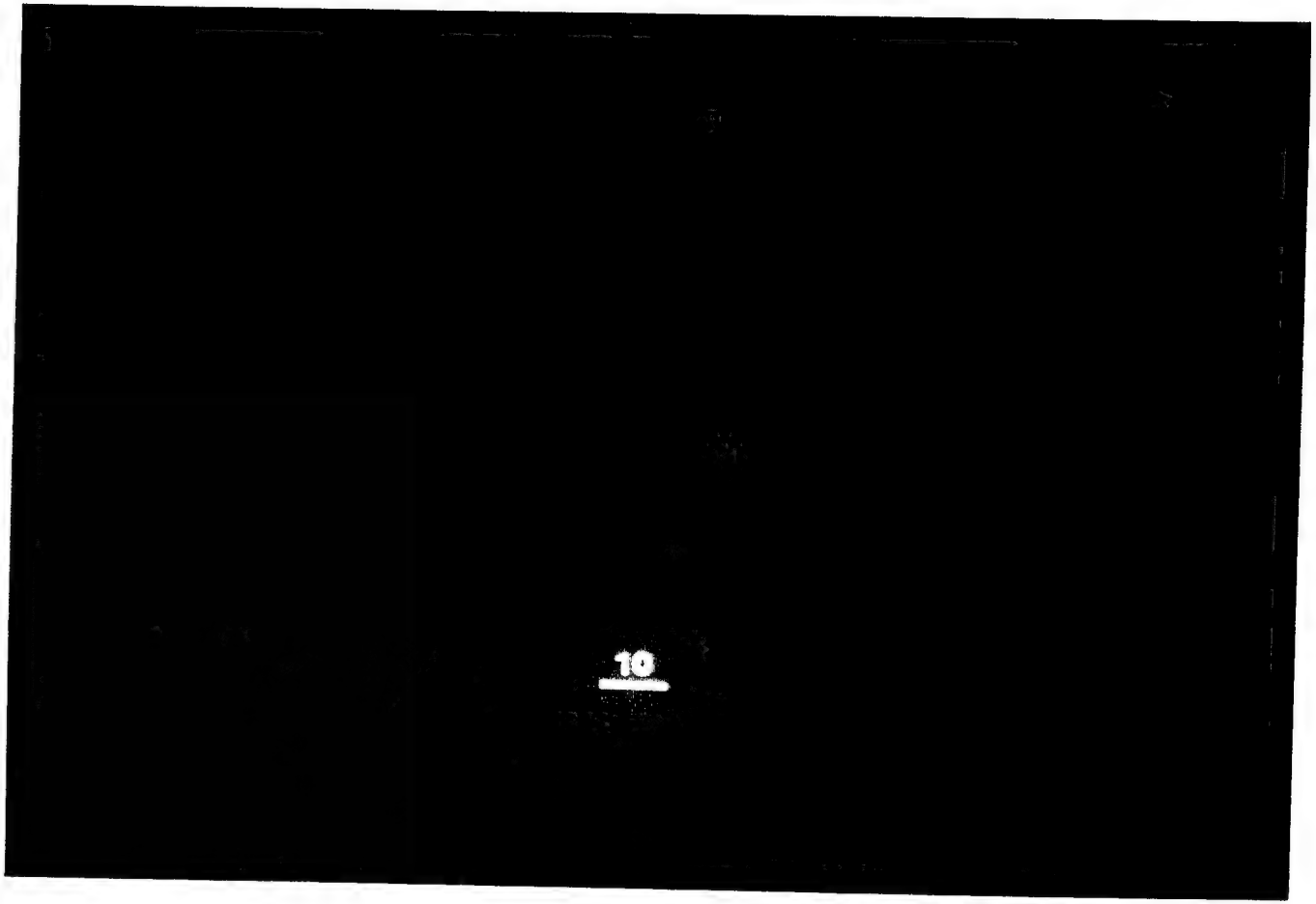


FIG. 14b Immunofluorescense staining of MMP-9 and of CD44 in MCF-7 breast cancer cell line using confocal microscope. The cells were stimulated with phorbol ester (50 ng/ml) in seum free media. Rabbit anti-MMP-9 IgG was used; followed by rhodamine-conjugated goat against rabbit IgG.(red color). Rat anti-CD44 IgG was used, followed by fluorescein-labeled (green color) goat anti-rat IgG. These analyses show that phorbol ester has stimulated the production of MMP-9 and CD44. MMP-9 is located more in the granules around the nucleus and less at the transmembrane.

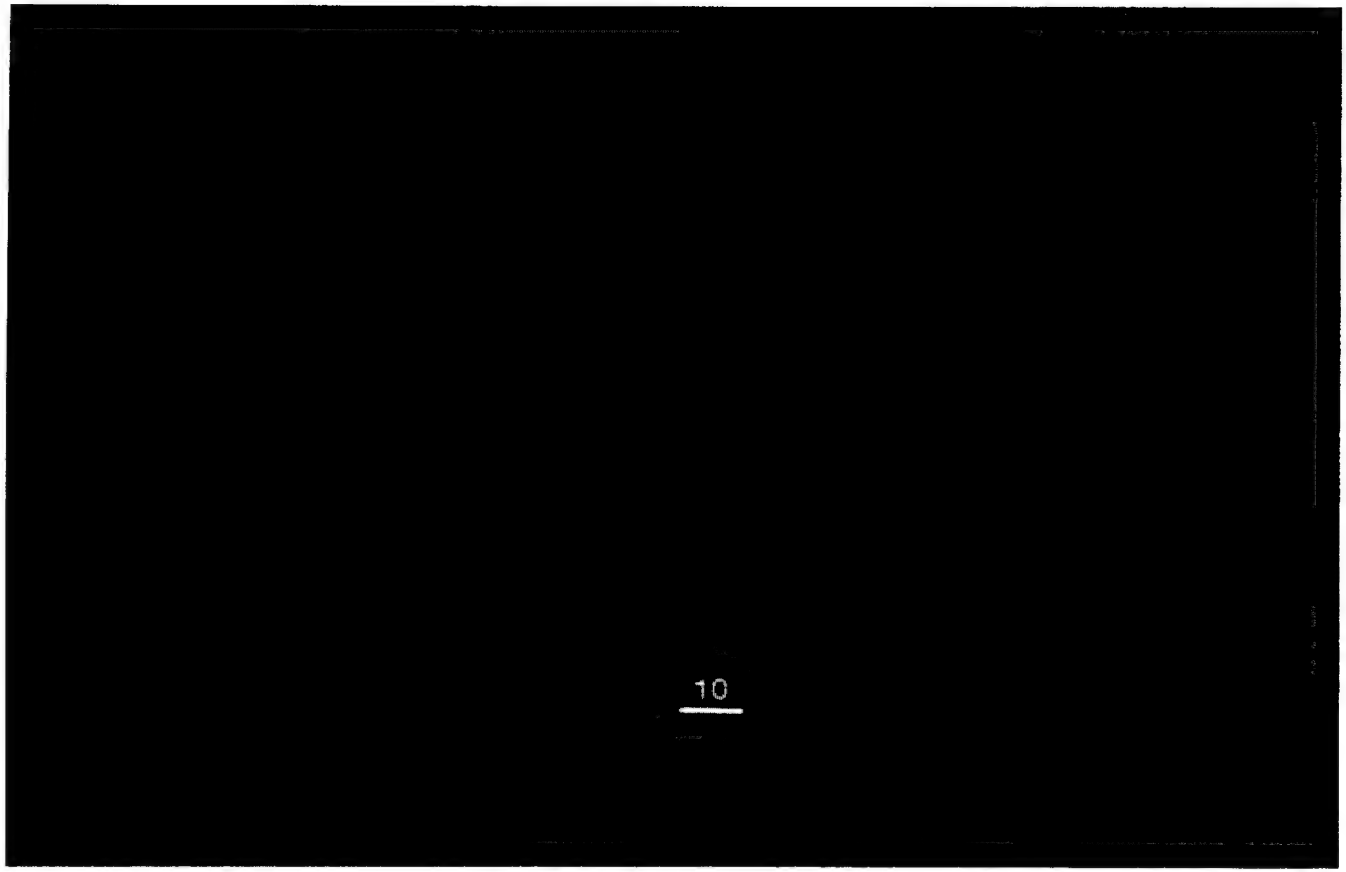


FIG. 15a Immunofluorescence staining of MMP-2 and of CD44 in MCF-7 breast cancer cell line using confocal microscope. The cells were in resting phase in serum free media. Rat anti-CD44 IgG was used, followed by fluorescein-labeled (green color) goat anti-rat IgG. Rabbit anti-MMP-2 IgG was used, followed by rhodamine-conjugated (red) goat against rabbit IgG. These analyses show that MMP-2 stains extremely weak and is found only near the cell surface as found for wekly stained CD44.

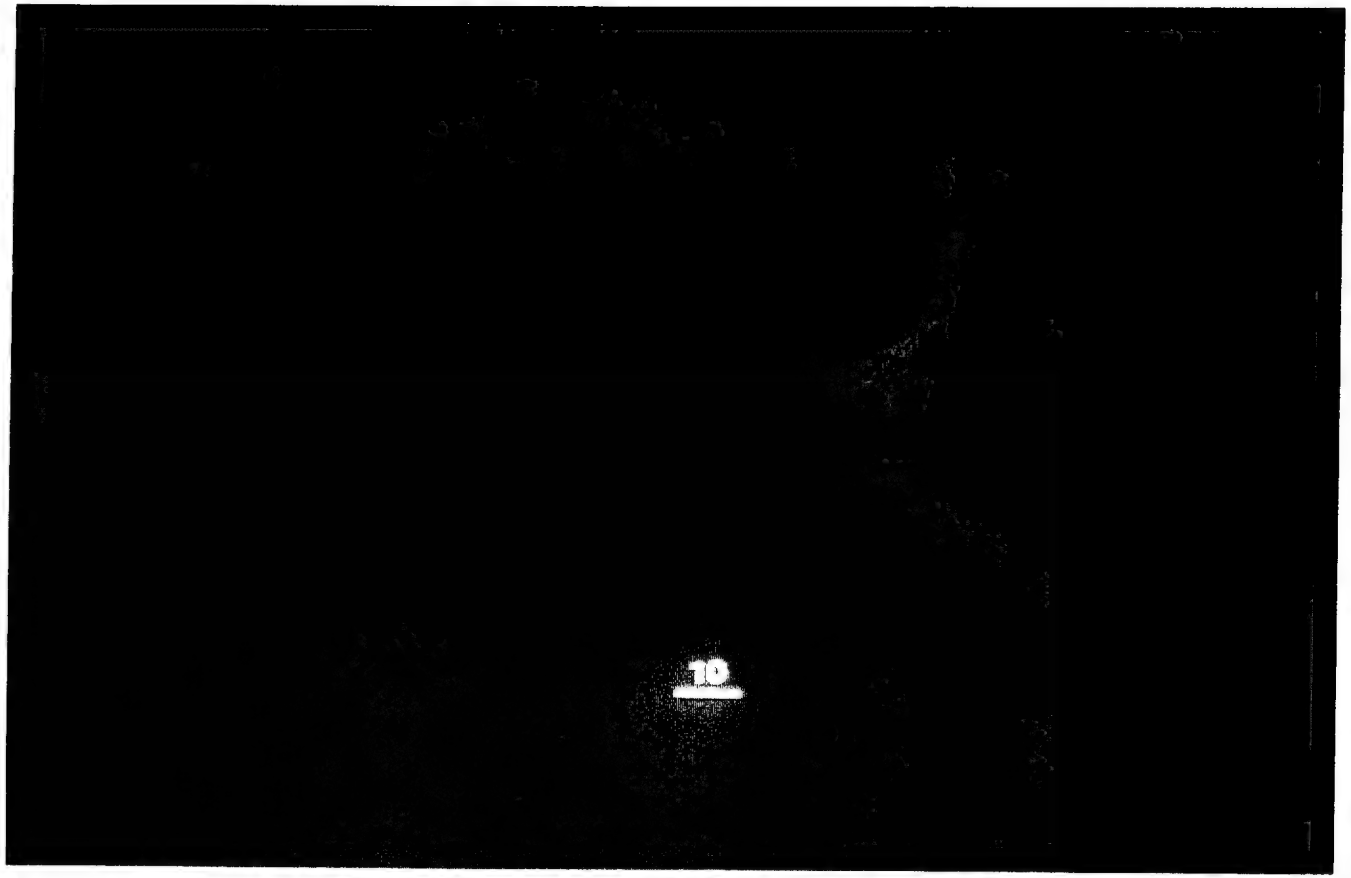


FIG. 15b Immunofluorescense staining of MMP-2 and of CD44 in MCF-7 breast cancer cell line using confocal microscope. The cells were stimulated with phorbol ester (50 ng/ml) in seum free media. Rabbit anti-MMP-2 IgG was used; followed by rhodamine-conjugated goat against rabbit IgG.(red color). Rat anti-CD44 IgG was used, followed by fluorescein-labeled (green color) goat anti-rat IgG. These analyses show that phorbol ester has stimulated the production of MMP-2 and CD44. MMP-2 although not secreted in the media (zymography) stains strongly and is found to be located in the transmembrane along with the stimulated production of CD44.

than that found in breast carcinoma tissues. Presence of stromelysin-1 or stromelysin-3 could not be demonstrated by zymography or substrate assays. Presence of stromelysin-3 can only be demonstrated by measuring mRNA levels [19], as the proteolytic activity of this enzyme appears to be very weak[20]. The measurement of mRNA levels of TIMPs and MMPs may be a necessary part of the either second or third year part of the present study.

Another important finding is that several tumor cell lines secreted MMP-9 and this secretion was enhanced by PMA stimulation of the tumor cells. TIMP-1 was also secreted in the similar fashion. TIMP-2 production was not enhanced in these cells. Mackay *et. al.*[47] observed similar stimulation of tumor cells by phobol ester and minimal stimulation of fibroblast cells. Future studies will include manipulation of tumor cells with protein kinase C (PKC) inhibitors, mainly staurosporine and by non-PKC-activating phobol ester

Meaningful statistical analysis of 50-100 samples are now feasible by the analysis of gelatinase MMPs by zymography and quantitation of MMP bands by use of GelBase/GelBlot Pro software. Storage of fresh frozen samples over five years and the availibility of information from Florida Tumor Registry of follow-ups of patients will allow us to fine tune the collected information. These findings may lead to the development of markers for early diagnosis in breast cancer.

Immunofluorescence staining and confocal microscope analyses of tumor cell lines has led to some important finding that either MMP-2 or MMP-9 are associated with CD44 (a transmembrane glycoprotein) isoforms in tumor cells. Preliminary results indicate that anti-CD44v-III isoform found in breast tumors activated the secreted MMP-9. MCF-7 cells do not secrete MMP-2 into the media as shown by zymography. Immunofluorescence staining with anti-MMP-2 IgG showed staining in the cell membrane suggesting the presence of MMP-2 bound to the membrane. Recently, an MMP has been identified as an integral part of plasma membrane protein [46, 48] and is termed as membrane-type protein (MT-MMP). This MT-MMP can activate MMP-2, which in turn can activate MMP-9 and other MMPs. MT-MMp has a dual role in assissting cell migration. It is membrane associated and can localize ECM digestion in the vicinity of the cell surface and also amplify the destruction of matrix by activating other MMPs. MCF-7 tumor cell line was shown to have high capacity to bind exogenous MMP-2 by use of radioreceptor -binding assay [49]. Our findings by confocal studies of MCF-7 cells for MMP-2 localization in transmembrane may sggest that MMP-2 made by the stimulated cells bind to TIMP-2, which in turn binds to MT-MMP [48].

There are several exciting findings in our present study that need to be expanded in the second year of the granting period. The study on analysing the breast tissues for MMPs and TIMPs will be an ongoing

project for second and third year and then correlate these findings obtained to be a part of the fourth year.

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APPENDIX

STATEMENT OF WORK

Role of Matrix Metalloproteinases and Their Tissue Inhibitors in Human Breast Adenocarcinoma

Task 1. Gather more histo-pathological information on well-characterized specimens that are available from the tissue procurement laboratory of Sylvester Cancer Center, Miami. Aproximately 25 specimens collected 4 years ago are available. Additional comparable specimens are available for each subsequent year.

a. Year 2: more immunohistochemical analysis with 4 other antibodies on 45-50 specimens for which pathology is known. Initially there will be 2 groups of tissues - in situ and invasive (± subsequent metastasis).

b. Year 3: consider further division of tissues into groups based on criteria suggested by initial studies - e.g., nuclear grade.

c. Year 4: collect data to complete all subdivisions.

Task 2. Collect more enzymological data and mRNA data.

a. Year 2: Analyze the same specimens for which immunohistochemical data are available. Analyze mRNA content by Northern blots or if necessary, by RT-PCR method.

b. Year 2: Also continue cell culture analyses and show a corelation between MMPs and isoforms of CD44 in defined breast cell lines.using confocal microscope. Such analyses to be correlated for breast tissues Define the role of MMP-2 binding to CD44 isoforms or to MT-MMP.

Task 3. Fresh tissues will be collected from patients for explant epithelial culture studies. This task will be initiated in the third year. Enzyme and inhibitor studies will continue into the final years.

Task 4. Correlate the findings obtained from the immuno-histochemical analyses, quantitation of MMPs and TIMPs and their mRNAs, pinpoint the over- or under-expression of MMPs or imbalance between the enzymes and inhibitors. Design further experiments to bolster or refute the underlying hypothesis. This task will be ongoing throughout the grant period, but adequate data will probably first be available in the final two years.

TABLE 1. Quantitation of Matrix Matalloproteinases--Gelatinases A and B and Interstitial Collagenase (MMP-1) in Breast Tissues.

Tissue	No.	GelatinasesA + B[+] Total Units	Collagenase[+] Total Units	Protein mg /g tissue	Tissue Wet Weight g
Normal T + 1M G	4	4.1	0.15	47.9	0.34
Benign T + 1M G	4	10.1	0.58	50.0	0.34
Adeno-carcinoma T + 1M G	11	98.1	5.5	55.1	0.23
Fibrocystic-disease T + 1M G	1	16.5	1.8	62.3	0.36

Gelatinase A + B = 72 kDa + 92 kDa gelatinases, Total Units = latent + active enzyme.
T = 0.25% Triton extracts; 1M G = 1M GuHCl extracts.
One enzyme unit = 1μg of substate digested /min at 37° C for gelatinases & at 30° C for collagenase.
[+] Values expressed as enzyme units/g wet weight breast tissue.

Table 2. Relative Amounts of Tissue Inhibitors of Metalloproteinases (TIMPs -1, -2 and -3) in 1M GuHCl extracts of Breast Tissues.

Tissue	No	TIMP-1	TIMP-2	TIMP-3
Normal	4	1.0	1.5	1.0
Benign	4	0.5	3.0	0.5
Adeno-carcinoma	11	1.0	2.0	0.5
Fibrocystic disease	1	0.5	2.5	0.5

Arbitrary Units:
1 = +
2 = ++
3 = +++

(9) APPENDIX

UNIVERSITY OF Miami
SCHOOL OF MEDICINE

September 13, 1995

Zeenat Gunja-Smith, Ph.D.
Department of Medicine
Univ. of Miami Medical School
Miami, Florida

Dear Zeenat,

I am writing to confirm my willingness to continue our collaboration by examining the tissue sections and cell lines with confocal laser scanning microscopy. This will provide precise information on spatial distribution and location of the various metalloproteinases and their tissue inhibitors in breast cancer. The project on the role of metalloproteinases in breast cancer complements our project on the role of CD44 in breast cancer.

Of particular interest is the fact that You have made some potent and specific polyclonal antibodies to various metalloproteinases and their tissue inhibitors. These antibodies together with ours to CD44 may shed some light on the interaction (if any) of CD44 and membrane metallo-proteinases in variuos transfected breast cancer cell lines and the cancer breast tissue.

Results from the collaborative studies will potentially have broad implications for potential early markers or for potential therapeutics for human breast adenocarcinomas.

Yours Sincerely,

Lily Bourguignon, Ph.D.
Professor of Cell Biology
and Anatomy

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